

Light and Electron Microscope Studies
of Host-Parasite Relations in a
Mycoparasite

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To my parents
who made it all possible.

ABSTRACT

Light microscope studies of the mycoparasite Piptocephalis virginiana revealed that the cylindrical spores of the parasite became spherical upon germination and produced 1-4 germ tubes. Generally two germ tubes were produced by each spore. When this parasite was inoculated on its potential hosts, Choanephora cucurbitarum and Phascolomyces articulatus, the germ tube nearest to the host hypha continued to grow and made contact with the host hypha. The tip of the parasite's germ tube became swollen to form a distinct appressorium. Up to this stage the behavior of the parasite was similar regardless of the nature of the host. In the compatible host-parasite combination, the parasite penetrated the host, established a nutritional relationship and continued to grow to cover the host completely with its buff colored spores in 3-4 days. In the incompatible host-parasite combination, the parasite penetrated the host but its further advance was arrested. As a result of failure to establish a nutritional relationship with the resistant host, the parasite made further attempts to penetrate the host at different sites producing multiple infections. In the absence of nutrition the parasite weakened and the host outgrew the parasite completely. In the presence of a non-host species, Linderina pennisporea the parasite continued to grow across the non-host hyphae without establishing an initial contact. Germination studies showed that the parasite germinated equally well in the presence of host and non-host species.

Further electron microscope studies revealed that the host-parasite interaction between P. virginiana and its host, C. cucurbitarum, was compatible when the host hyphae were young slender, with a thin cell wall of one layer. The parasite appeared to penetrate mechanically by pushing the host-cell wall inward. The host plasma membrane invaginated along the involuted cell wall. The older hyphae of C. cucurbitarum possessed two distinct layers of cell wall and showed an incompatible interaction when challenged with the parasite. At the point of contact, the outer layer of the host-cell wall dissolved, probably by enzymatic digestion, and the inner layer became thickened and developed a papilla as a result of its response to the parasite. The haustoria of the parasite in the old hyphae were always surrounded by a thick, well developed sheath, whereas the haustoria of the same age in the young host mycelium were devoid of a sheath during early stages of infection. Instead, they were in direct contact with the host protoplast. The incompatible interaction between a resistant host, P. articulatus and the parasite showed similar results as with the old hyphae of C. cucurbitarum. The cell wall of P. articulatus appeared thick with two or more layers even in the 18-22 h-old hyphae. No contact or interaction was established between the parasite and the non-host L. pennisporea. The role of cell wall in the resistance mechanism is discussed.

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CHAPTER 1INTRODUCTION

The nature of resistance in host plants to their parasites is one of the major biological concerns that is not fully understood to-date. Moreover, for obligately biotrophic pathogens, the mechanism behind such host resistance, and the knowledge of the host-parasite interaction is rather limited. However, knowledge of the specific process involved in host's resistance may not only provide information about the way in which host-parasite specificity is determined, but also hold some promise for more precise and long lasting control of plant pathogens in future.

The questions of recognition and determination of host specificity of the biotrophic fungi are among the most important logical steps towards the elucidation of such host-parasite relationships (Manocha and Golesorkhi, 1979). As a parasite establishes contact with a host species, a complex series of interactions occur which determine whether a functional relationship will develop between host and parasite, and what the outcome of that relationship will be. In fact from the myriad parasites that may come into contact with the surface of a particular host, only a minute proportion have the ability to penetrate, become established and multiply within the host tissue. Generally some parasites are able to attack a wide variety of host species. In other parasites, especially

the biotrophic ones, the close association of the host-parasite relation has led to the establishment of specific inter-relationships in which the parasite is able to grow only in association with a particular host species.

The genus Piptocephalis of order Zoophagales, class Zygomycetes is comprised of biotrophic, haustorial mycoparasites of mucoraceous fungi. These mucoraceous fungi usually develop on the dung of herbivorous animals and it seems that Piptocephalis species may be of common occurrence in soil to parasitize these fungi. However even among the Mucorales all members are not equally susceptible to infection by P. virginiana, and some are completely resistant, e.g., Phascolomyces articulatus a member of family Thamniaceae of order Mucorales. In fact, P. articulatus has been described by Jeffries and Young (1978) as a useful experimental organism for studies on the fine structural aspects of mechanism of resistance to infection, when challenged with species of Piptocephalis, e.g., P. unispora. On the other hand, Choanephora cucurbitarum, a member of choanephoraceae of order Mucorales is a susceptible host when young but becomes resistant with age. Berry (1959) recorded similar observations on Helicostylum species parasitized by Piptocephalis virginiana. Moreover Linderina pennispora a zygomycetes fungus of order kickxellales, is a non-host species which has not been reported to be parasitized by the Piptocephalis species. (Jeffries and Young, 1978).

The ability of P. virginiana to initiate the process of infection of P. articulatus and aged C. cucurbitarum, coupled

with its inability to sustain active growth on these potential hosts, seemed to provide a suitable means for comparing the fine structural aspects of resistance to mycoparasitic infection with the previously described system of C. cucurbitarum (Manocha and Lee, 1971, Manocha and Letourneau, 1978) in which the host was actively parasitized when young. Therefore the need for further information on the structural and cytological events leading to the establishment or rejection of P. virginiana by its potential hosts and non-hosts, appeared to be pertinent for the understanding of the host-parasite interactions, host specificity and mechanisms of resistance in the haustorial mycoparasites; hoping that these studies would provide further basic insights that could be applied to the more complicated plant-fungus systems.

The present study is in main electron microscopy in order to define precisely the events in the interaction of the host-parasite relationship. Ultrastructural changes in the young and old host species of C. cucurbitarum, P. articulatus and non-host species L. pennisporea when challenged with P. virginiana are described. Supportive evidence by the use of light microscope are forwarded, along with observations on the parasites germination when inoculated with each host and non-host species. Moreover, precise investigations of the morphology of infection is an indispensable first step towards biochemical and biophysical definition of parasitism and host resistance.

CHAPTER 2LITERATURE REVIEWI Mycoparasitism

Knowledge of host-parasite relationship between plants and microorganisms is essential to plant pathology, however the process of obtaining the knowledge has been rather slow and tedious due to the complicated nature of this relationship. Few questions have been resolved regarding the two main areas of plant pathology i.e., the nutritional requirements of the parasite (or basis of parasitism) and the cellular and molecular basis of disease resistance. This being attributed to the highly complex nature of vascular host plants (Barnett and Binder, 1973). Most studies on host-parasite relationship at a cellular level mainly pertain to fungi parasitic on higher plants (Ehrlich and Ehrlich, 1963; Peyton and Bowen, 1963; Shaw and Manocha, 1965). These studies were mostly concerned with the structure of the haustorial apparatus and the interpretation of the interface (Bracker, 1967) and have not furnished us with an answer to problems such as mechanism of parasitism and nature of disease resistance. It is most probable that the complexity of the host plant has likely obscured the true picture. Rarely has a mycoparasite (the phenomenon of one fungus parasitic on another fungus) been an object of investigation (Armentrout and Wilson, 1969). Only recently the study of mycoparasitism has provided a new horizon for elucidation of the basic principles underlying

parasitism and resistance, owing to certain advantages they have over the more complicated plant parasite system (Barnett and Binder, 1973). Namely, their ease in culturing, rigid control of the host nutrition, relative simplicity of the host cell and the apparent interface similarity with those of plant-fungal systems (Manocha and Lee, 1971).

Much of the earlier research on mycoparasites was based on taxonomy, host ranges, nutritional requirements, cultural conditions and on the different modes of infection. Such investigations have been reviewed by Boosalis, 1956; Barnett, 1964; Madelin, 1968; and Barnett and Binder, 1973.

Recently mycoparasites have been divided into two groups based on their mode of parasitism: Necrotrophic and biotrophic. The necrotrophic parasite contacts its host, excretes toxic substances which results in the death of the host tissue and derives the released nutrients from the dead host cells (Barnett and Binder, 1973). Biotrophic fungi are defined as those which establish an intimate association with the compatible host organelles, drawing sufficient nutrients to support their growth but not drawing up host energy supplies to the point that death ensues before they have an opportunity to multiply (Sequeira, 1979). Barnett and Binder (1973) have categorized these biotrophic mycoparasites into 3 distinct groups on the basis of their morphology and physiology;

- 1) The internal mycoparasites; which are represented by chytrids grown within the cells of other fungi.

- 2) The contact mycoparasites; the fungi imperfecti are placed in this category. They do not produce haustoria or internal hyphae.
- 3) The haustorial mycoparasites; are members of order Mucorales. Characteristically they produce distinct haustoria within the host hyphae. The last group of haustorial mycoparasites are the major focus of this study.

Seventy species of filamentous, biotrophic, mycoparasitic fungi which have been known to produce haustoria within the hyphae of their host are all merosporangial members of the mucorales i.e., produce spores in rod like sporangia (Benjamin, 1959, 1961; curtis et al, 1978). With few exceptions their host range is limited to the same order, Mucorales. Depending on conditions they may have no effect on their host's growth, cause stimulation, or an inhibition. Occasionally, however, morphological disturbances do occur in the host (Curtis et al, 1978). The principal genera are Syncephalis and Piptocephalis in the family piptocephalidaceae of order Zoophagales and Dispera, Dimargaris and Tieghemiomyces in the family Dimargaritaceae of order Dimargaritales (Barnett and Binder, 1973). Recently interest has developed in investigating different aspects of mycoparasitism including nutrition, physiology and ultrastructure that may be relevant to the basis of this kind of host-parasite relations (Barnett, 1964, 1970; Manocha and Lee, 1971, 1972; Barnett and Binder,

1973; Binder and Barnett, 1974; Jeffries and Young, 1975, 1976, 1978; Manocha, 1975; Manocha and Deven, 1975; Phipps and Barnett, 1975; Barnett and Pierce, 1976; Manocha and Letourneau, 1978; Manocha and Golesorki, 1979). However, it should be noted that one of the most extensively studied mycoparasite both at the ultrastructural and biochemical level is Piptocephalis virginiana.

II Mode of Parasitism

In order to obtain the required nutrients for growth and reproduction it is necessary for parasitic fungi to enter their hosts and establish direct contact with them. The nature of penetration of the host cell wall by the parasite is important since it is the first step towards the establishment of the host-parasite relationship. The infection process of biotrophic haustorial mycoparasites usually appears to follow a consistent pattern when examined at an ultrastructural level. The most important stage in the life cycle of a biotrophic fungus occurs during the time immediately following the germination of its spores upon the surface of a potential host (Ingram, 1976). During this period a number of characteristic specialized infection structures are formed (Bushnell, 1972). The function of their structures is to bring the fungus from the host surface where its spores germinate to an area favourable for haustorium formation. The germinating spore on a potential host produces a germ tube which comes into contact with the host cell surface. The parasite's germ

tube then contacts the host cell followed by the formation of a specialized structure the appressorium, which must form prior to the actual penetration if infection or at least penetration is to occur. These appressoria are adhesive discs securing a parasite fungus to its host (Yarwood, 1956). The initiation, formation and action of the appressorium are essential parts of the infection process of many fungi (Emmett and Parbery, 1975). Formation of haustoria is the next stage of development in the host-parasite interface, which is the enlarged penetration peg after its ingress into the host cell wall. Haustoria are considered to be uniquely parasitic specialized outgrowths of fungus cells which due to their intracellular location are considered as feeding structures (Aist, 1976a). These structures differ greatly in both gross and fine structural anatomy depending on the particular parasite in question (Barnett and Binder, 1973). The haustorial apparatus of many of the haustorial mycoparasites morphologically consists of an appressorium, a neck region with a neck ring and a small papillae or collar, and a lobed region surrounded by a sheath matrix enclosed in an extrahaustorial membrane (Jeffries and Young, 1976).

III Mechanisms of primary penetration: Enzymatic or Mechanical?

The question whether penetration of the infection peg occurs via mechanical means or enzymatic dissolution of the host cell wall has long been a matter of contention. Earlier literature on this topic suggest that since biotrophic

fungi cause little structural damage in their hosts, they have a limited capacity for production of extracellular wall degrading enzymes and that wall penetration is therefore achieved via mechanical pressure (Ingram et al.,1976). The fact that the appressorium attaches firmly to the host cell wall by a secretion of the fungus, and provides the required adherence against which the infection peg might generate the force required for penetration (Dickinson, 1960) lent support to this mechanical hypothesis, along with the demonstration that fungal infection pegs can penetrate biologically inert barriers such as gold foil (Brown and Harvey, 1927). Akai et al (1968) observed cracks in the cell wall of barley leaves intersecting the infection peg of Erysiphe graminis, suggesting that a large amount of mechanical pressure was being exerted. Manocha and Lee (1971) reported that P. virginiana mycoparasitic on C. cucurbitarum involved mechanical means for host cell wall penetration, however enzymatic dissolution of host cell wall was not ruled out by them in this system. Politis and Wheeler (1973) and Stanbridge et al (1971) reported that the cuticle may be curved inward around the penetration pore during growth of the penetration peg through the host wall. Aist and Williams (1971) suggest that the penetration of the walls of Brassica root hairs by the stachel of Plasmodiophora brassicae may be totally mechanical. Finally Bushnell (1972) suggests that regardless of the degree of dissolution of cuticle and wall, some mechanical force is required for penetration of the infection peg.

Considerable evidence is now available which suggests that the host cell wall may be enzymatically degraded during penetration and that most biotrophic fungi do possess the appropriate enzymes for cell wall degradation (Aist, 1976a). Kunoh and Akai (1969) and McKeen et al (1969) along with rather sufficient biochemical evidence have shown that the halo of host wall alteration around the infection peg of E. graminis contains reduced amounts of cutin, polysaccharide (including cellulose) and pectin, and increased amounts of reducing sugars and pentose and uronic acids. This is an indication of a partial degradation of the host cell wall during the penetration process by these fungi. McKeen (1974) reported that the cuticle of Vicia fabia seemed to be enzymatically dissolved by the fungus Botrytis cinerea. The penetration peg of this fungus, formed sharp clean penetration pores with no obvious signs of physical deformation of the host cuticle. Esterase activity was observed in the tips of the germ tube of this parasite. Stanbridge et al (1971), and McKeen and Rimmer (1973) reported that the tip of the penetration peg of E. graminis may have an irregular outline as it presumably passes through the host wall which suggests that it may be filling a space formed by the digestion of host wall microfibrils and that the penetration pegs of E. graminis f. sp. hordei presumably fixed during development may be completely or partially devoid of a wall, which could make a purely mechanical penetration unlikely. Transitory esterase activity has been reported for Venturia inaequalis a fungus

which ramifies between the cuticular membrane and the cell wall by Nicholson et al (1972). In the infection of lettuce epidermal cell by Bremia lactucae, the infection peg penetrates the cuticle outer zone of the wall, leaving the inner zone intact. The profiles of the cuticle and wall microfibrils are not distorted and no indication of mechanical pressure is observed. Ingram et al (1976) suggest that B. lactucae and other biotrophs are capable of enzymatically dissolving the host cell wall. Edwards and Allen (1970) support the idea that both modes of cell wall penetration are necessary. They reported that during the infection of barley by E. graminis penetration consists of both enzymatic digestion of the cellulose portion of the epidermis and the mechanical pushing of the infection peg through the papillae. Manocha and Golesorkhi (1979) do support the concept that the penetration of P. virginiana on mature hyphae of C. cucurbitarum seemed both enzymatic and mechanical by the dissolution of the outer layer at the point of contact and by the mechanical push through the inner layer of host cell wall. Penetration of the host Cokeromyces recurvatus by the infection peg of Piptocephalis unispora involves both mechanisms also. Wall material of the host is both eroded and depressed by the infection peg and then breached (Jeffries and Young, 1976). It can be clearly realized that there is rather good evidence for both an enzymatic and mechanical mechanism of penetration. There is probably no one pathogen for which penetration has been proven to be purely mechanical or enzymatic. In the few instances

where both mechanism have been thoroughly studied there is good reason to accept that a combination of both systems i.e., mechanical and enzymatic degradation have been involved (e.g., powdery mildews) (Aist, 1976a). Further investigation of the nature of penetration will require more elaborate biochemical studies which is not only of importance to revealing the mechanism of penetration alone, but in understanding the mechanism of specificity too, since Albershim et al (1969) suggested that it may be through the interaction of wall degrading enzymes and their substrates that specificity may be determined.

IV Fine structure of host-parasite interface

Earlier investigations on the host-parasite interface have placed emphasis on the fine structure of the haustoria of obligate parasites, however interfaces in various host-parasite combinations have been recently reviewed at length by Bracker and Littlefield (1973). It has been observed by all fungus haustorial pathogens investigated to date, that although the host cell wall is breached, the host plasma membrane around the haustorium is invaginated but remains intact during early stages of infection to accommodate the parasite (MSc. Thesis Letourneau, 1978; Abu-zinada et al, 1975; Littlefield and Bracker, 1970, 1972 and Bracker, 1968). The extrahaustorial membrane is generally acknowledged as the invaginated host plasma membrane which is invaginated by the advancing haustorium. This membrane is different in

characteristic from the host plasma membrane (Littlefield and Bracker, 1972). These investigators noticed that the extrahaustorial membrane had different staining characters and dimensions than the plasma membrane and was non granular as compared to the granular appearance of the host plasma membrane (Littlefield and Bracker, 1973). At early stages of infection the extrahaustorial membrane appears to be closely appressed to the fungal wall, and as the infection process proceeds a separation develops between the membrane and thehaustorial cell wall (Shaw and Manocha, 1965). A distinct electron-dense zone then appears between thehaustorial cell wall and extrahaustorial membrane and this zone is referred to as sheath encapsulation (Manocha and Letourneau, 1978). Thehaustorial sheath is equivalent to the encapsulation shown by Heath (1972), Heath and Heath (1971), Ehrlich and Ehrlich (1963), Shaw and Manocha (1965), Kunoh and Akai (1969) and the zone of apposition Chou (1970), and Peyton and Bowen (1963). This matrix of the sheath is the environment in which ahaustorium exists and the medium that coats thehaustorium. The sheath seems to be a conceptual common denominator of allhaustoria, usually of different size and extent (Bracker and Littlefield, 1973). The composition of this sheath is believed to be flexible and in most cases may vary from a liquid or solution to thickened viscous material or gel (Bushnell, 1972; Zimmer, 1970; Hanchey and Wheler, 1971; Manocha, 1975).

V Origin of the haustorial sheath

The origin and biochemical nature of the haustorial sheath has long been matter of controversy. Owing to its accompanying most host-parasitic combinations studied so far, it has been a focus of attention and different workers have interpreted its origin and composition differently (Bracker, 1967). Some investigators suggest its origin from the host, others from the parasite and still others believe the sheath to be composed of by products from both the host and the parasite. Most of these interpretations of the sheath origin are based on visual observations of the electron micrographs, with little supportative biochemical evidence for any of these views (Manocha and Letourneau, 1978). As early as 1900 Smith suggested that the sheath was debris from the host wall. Some of the other investigators supporting this view that they were of host origin, derived from the protoplast are Berlin and Bowen (1964), Peyton and Bowen (1963) and Shaw and Manocha (1965) particularly since membrane profiles resembling secretory components were seen at the periphery of the sheath. Peyton and Bowen (1963) noticed vesicles around the haustorium of Peronospora manshurica and believe these are secretory bodies derived from the host cytoplasm and fusing with the plasmalemma of the host cell to discharge material into the sheath matrix. Shaw and Manocha (1965) also observed numerous vesicles and endoplasmic reticulum in the host cell close to the sheath and suggest it could be related to the production of sheath since their abundance was not observed in uninfected

cells. It has been suggested that the presence of numerous vesicles and extensive development of endoplasmic reticulum may be associated with the formation of the sheath (Manocha, 1966; Manocha and Lee, 1971). ABu-zinada et al (1975) report that the haustoria of Uromyces fabae were usually surrounded by a sheath membrane continuous with the host plasma membrane, and reacted like host cell walls to the electron stain. Armentrout and Wilson (1969) suggest that the sheath is composed of material contributed by both host and parasite and pointed out "blebs" from the sheath, which appears to release material from the parasite via the matrix of the sheath into the cytoplasm of the host. However Heath (1972), Mckeen et al (1966) and Zimmer (1970) support the idea that sheath is a result of the host-parasite interaction and is maintained by continuous interaction. Ehrlich and Ehrlich (1963) attributed the origin of the haustorial sheaths in the wheat rust to the cytoplasm of the haustorium and they noticed continuity of the haustorial cytoplasm through small channel areas in the haustorial wall to the sheath. Chou (1970) and Hardwick et al (1971) however, suggest the sheath as being part of the fungal wall.

VI Role of haustorial sheath in host-parasite interactions

The extrahaustorial sheath might have an effect on the host-parasite relationship, yet, the exact function of this structure in development, and in resistance-suceptibility reactions is not uniformly agreed upon. Considerable evidence

is available to demonstrate correlations existing between the nature of the sheath and age or host parasite compatibility (Bracker and Littlefield, 1973). Generally the formation of the haustorium is opposed by many chemical and physical barriers in the host, some preformed, others like the papillae and sheath produced in response to attack. A parasite grows without interruption whenever the host is compatible, but growth is ceased or retarded at different stages if they are incompatible (Bushnell, 1972). The sheath is a common structure and can be thought of as a normal response to haustorial fungi and to other plant pathogens if the host is still alive after the initial penetration (Chou, 1970). Hence these structures are most likely non-specific responses to wounding attributed by the host and that successful penetration of some pathogens is owing to the haustorial parasite to have a means to limit this response (Bushnell, 1972). The extrahaustorial sheath may play a role in host-parasite relations. Heath and Heath (1971) in a study of comparison of the susceptible and immune reactions of cowpea leaves to rust infection showed that signs of incompatibility were detected in the immune variety during early stages of haustorial formation by the enclosure of the haustorium in a callose containing sheath, whereas no such callosity was seen in the susceptible cowpea leaves. The encapsulation around the young haustoria in the susceptible host was mainly electron transparent (at 26 h), however in 6 day old infections, a few haustoria were found enveloped in thick sheaths, similar to those observed in the immune reaction. They suggest, the sheath formation could be one

form of response to haustorial formation in restricting the pathogen in absence of hypersensitive response. (Heath (1977) further reported that one way in which some non-host respond to infection to rust can be achieved by formation of electron-opaque material on and within surrounding host cell wall and consequently prevention of haustorial formation. In all non-hosts examined, uncased haustorial when observed, were accompanied by eventual collapse and darkening of invaded cells. Zimmer (1970) reported that the sheaths surrounding the haustoria of incompatible hosts were more amorphous than in a compatible host. ABu-Zinada (1975) found that the haustorium of Uromyces fabae was surrounded by a sheath of unknown material. In the zone at the base of the haustorial neck and beneath the host cell wall a layer of fibrillar material forming a collar was seen. This material reacted like the host cell walls to the electron stain. They believe this structure to be a defensive response to penetration and to support their view they report the presence of necrotic haustoria completely walled off by material similar and continuous with that of the collar, thus attributing the death of such haustoria to the exclusion or restriction of the exchange substances between host and parasite. In case of infection of lettuce, callose is deposited as an extension of the host wall to form a collar around the developing haustoria of B. lactucae. As the haustoria mature this deposit may extend and in most cases finally completely enclose the haustorial body, as reported by Ingram et al (1976). They

associate complete encasement of haustoria with certain types of host resistance. Ehrlich and Ehrlich (1970) report the sheath to be larger but with reduced amounts of electron dense material around young haustoria as compared to the mature one. Bushnell (1972) concludes that the exact function of the extrahaustorial matrical material is unclear, as to whether they serve as a barrier to movement of substances, facilitate such movements or are unrelated to interchange of substances between the host and parasite.

Autoradiography techniques have been utilized recently to reveal the role of the sheath in host-parasite relationships. Ehrlich and Ehrlich (1970) inoculated host wheat leaves with prelabelled ^{14}C uredospores and demonstrated the passage of radioactivity from the parasite to the chloroplast and cell wall of the host. No label was observed in the sheath zone, indicating the passage of material from haustorium to host through the sheath. Mendgen and Heitefuss (1975) inoculated bean leaves with uredospores of Uromyces Phaseoli previously labelled by feeding the host with ^3H orotic acid. Their autoradio showed heavy labelling of the fungal structures including the fungal walls and haustoria, but in the sheath around the haustorium and host cytoplasm no label was viewed. These results are contradictory to the findings of Ehrlich and Ehrlich (1970) who concluded the migration of label from parasite to the host. Mendgen and Heitefuss (1975) attribute this to a result of non-specific diffusion of ^{14}C from the parasite to the host. Regardless, both studies show that the sheath zone does not act as a sink

for the radioactive materials released by the parasite, even though differences do exist among the two studies. Manocha (1975) in a study of the host parasite interfaces in wheat carrying the temp sensitive Sr6 allele for resistance to race 56 of Puccinia graminis tritici found that labelled leucine was seen in the haustoria of P. graminis at earlier stages of infection. At later stages of infection in both susceptible and resistant hosts a reduction in the amount of label incorporated was seen. The uptake of ^3H leucine into the haustoria stopped when the label was fed after 12 days in the susceptible host and after 4 days in the resistant host. These times are coincidental with the time of sheath formation. He suggested that the sheath developed earlier around the incompatible host-parasite combinations than in the compatible (susceptible) combinations. These results were in accordance and later supported by Coffey (1976) who investigated the fine structure of the major gene resistance involving the flax K gene and the rust fungus Melampsora lini. He reported that in resistant cells, there was a progressive increase in fibrillar material in the extrahaustorial matrix (sheath) at only 4 days after inoculation. However the matrix of the susceptible host remained electron lucent and the sheath did not develop until much later stages of infection. Manocha (1975) and Coffey (1976) do support the idea that a possibility exists that the earlier development of the sheath zone could function as major determinant in the resistance of the host to its parasite. Other studies (Shaw and Manocha, 1965; Manocha, 1966; Manocha and Lee, 1972) also do indicate that the

earlier development of sheath in incompatible host-parasite combinations may function in resistance rather than act as a nutritional sink.

VII Papillae

Over a century ago deBary noticed localized apparent wall thickenings on the inner surface of host plant cells at regions of penetration by fungi, and an existing correlation between their occurrence and the failure of further fungal growth. Recently these depositions have been seen to be of common occurrence and although not ubiquitous, they seem to accompany every host wall penetration in some host-parasite combinations (Aist, 1976b). If the growth of the intracellular fungus is minimal or not present at potential or actual penetration site, these depositions are usually hemispherical. However if considerable growth has occurred, they may conform to some extent to the intracellular parasitic structure around which they form and finally encase them (Aist, 1976b). The term sheath, has rather been misused occasionally to refer to papillae, but it has a different meaning in recent literature. A sheath is a rather extensive structure encasing the whole body of an advancing haustorium, being deposited between the host's extrahaustorial membrane and the fungal or haustorial cell wall. Whereas a papilla (also called a lignituber or callosity) is a heterogenous paramural deposition external to the host cell protoplast at the site of fungal intrusion (i.e., located between host cell wall and host plasma membrane).

(Aist, 1976b). Chemical stimuli are considered to incite papillae formation when physical stress is not present. This being evidenced by the directed cytoplasmic aggregation and papillae formation of plant cells close to those being penetrated, and also papillae formed prior to the penetration of host cell wall. The time of host wall penetration and papillae development often closely coincide. However the rapidity and duration of the deposition process is different according to different host-parasite combinations (Aist, 1976b). Callose and lignin have been identified to be part of the main constituents of papillae however cytochemical tests have shown major chitin-chitosan portion in papillae of fungal host Phycomyces blakeslee. Hence papillae are rather different from normal cell walls but their chemical constituents may reflect a degree of dependence on normal cell wall physiology, lending support to the idea that they may be of plant cell origin (Aist, 1976b).

VIII Function of Papillae

Different hypothesis regarding the function of papillae in host-parasite combinations have been forwarded. The most valid and evidenced views are the following. Papillae formation is one manifestation of wound-healing process that plant cells are capable of performing, or that it is probable that papillae are merely developed in response to (i.e., as a result of) penetration by parasite (Aist, 1976b). However observations of Smith (1900) stating that "Papillae formed in

response to penetration, retards the progress of the infection peg" has led into forming of one the most popular hypothesis regarding its function (Aist, 1976b). This view demonstrates different situations in which the incidence, structure and size of papillae relates to unsuccessful penetration attempts, and not with successful penetration by the fungi. To date, scant unequivocal evidence for the function of papillae in resistance exists, mainly due to a lack of detailed knowledge of their composition, the timing of their formation and the ability of their components to prevent fungal growth (Ride and Pearce, 1979).

During the course of an investigation of the host range of Piptocephalis unispora it was observed that although the parasite attacks germ tubes of Pascolomyces articulatus, further development of P. unispora is apparently so arrested that the mycelium of P. articulatus invariably outgrows that of the parasite (Jeffries and Young, 1978). This situation was contradictory with the interaction of P. unispora and Cokeromyces recurvatus, in which the host was actively parasitized and usually completely overgrown by the parasite (Jeffries and Young, 1976). Ultrastructurally, in the interaction between P. articulatus and P. unispora, papilla formation was associated with most sites of appressorial contact. Jeffries and Young (1978) believe the reason for inhibition in parasitic growth of P. unispora on P. articulatus could have been due to the formation of papillae which prevents the establishment of an active haustoria. They further

suggest the formation of papillae to be a wound response by the host which acts mainly by formation of a complete wall around the haustoria and therefore is a result of a physiological and/or biochemical resistance mechanism rather than their cause. Therefore, its function as a resistance factor is only to wall off indirectly the degenerative haustoria. Aist and Israel (1976) studied E. graminis and Olpidium brassicae on their respective host Hordeum vulgare and Brassica oleracea. They observed that in most cases in these systems papillae formed too late to present a mechanical barrier to the fungus. They further discuss the fact that certain parasitic units cease development before or during penetration in the complete absence of papillae. They indicate that those systems which cease development even in the presence of papillae do so due to similar non papillae related factors. They finally conclude that papillae are capable of offering "some" degree of resistance to the host. Several lines of evidence indicate that the process of papillae formation provides a mechanism for resistance to penetration in reed canarygrass leaves (Sherwood and Vance, 1967; Vance and Sherwood, 1977). The appositions contained liquified wall material and penetration pegs usually did not pass through them. However treatment of leaves with cycloheximide prevented protein synthesis, papillae formation and resistance to different non-pathogens of reed canarygrass, suggesting that resistance in incompatible plant-fungus combinations may involve papillae formation. More recently Aist et al (1979) used E. graminis, to examine its capability

of haustorium production in compatible barley cells at sites of preformed papillae. Using $\text{Ca}(\text{H}_2\text{PO}_4)_2$ on inoculated coleoptiles, oversize papillae were formed. They noted that the preformed papillae, appeared to allow few or no haustorium production of the appressoria, depending on the experimental design. They suggested that preformed papillae can prevent appressoria from haustorial formation in cells of a compatible host plant. However they finally concluded that resistance in plant-fungus combinations may involve papillae formation, but results from such experiments depends greatly on the condition of the experiment and on the nature of the host-parasite used and should not be interpreted to other conditions or combinations and that further critical experimental work is required to link such resistance more specifically to papillae formation.

IX Ultrastructural Studies of Mycoparasitism by Piptocephalis virginiana

To date, one of the most extensively investigated mycoparasites studied both ultrastructurally and biochemically is P. virginiana. The fine structure of the haustorial formation of this mycoparasite, infecting Mycotypha microspora was studied by Armentrout and Wilson (1969). They observed that upon infection in the host cytoplasm there was an increase in tubular endoplasmic reticulum and mitochondria, but fewer spherosomes were present as compared to the healthy host cells. They also observed the haustoria of mature P.

virginiana as being encased by an electron dense sheath with a convoluted surrounding membrane. They suggested a two-way exchange through the sheath matrix which was composed of materials originating from both the host and parasite since spherosomes were seen moving down the hyphae of the parasitic fungus towards its haustoria. They concluded that spherosomes of the parasite could supply enzymes to the sheath which gave a positive acid-phosphatase test. These enzymes could be released into the host cytoplasm via "blebs" that were seen in the sheath matrix. They further suggested that the endoplasmic reticulum was responsible for transport of material from the host, since their presence was seen in the vicinity of the haustorial sheath at the time of sheath development. Rosenthal (1970) questioned the integrity of their study, thus making the interpretations of Armentrout and Wilson (1969) dubious.

Mycoparasitic system of P. virginiana was chosen by Manocha and Lee (1971) to study its host-parasitic relations utilizing Choanephora cucurbitarum as the potential host for this system. The cell wall of the young host hyphae was breached by the infection peg, which at later stages of infection was elongated and formed the haustorial neck. The latter is surrounded by a collar, that appeared to be an extension of the host cell wall. During early stages of infection the haustorial cell wall was closely appressed to the host cell wall. A thin electron transparent zone was viewed around the haustorium at 20-22 hours after infection. This region was encased by the extrahaustorial membrane which at no time was breached by the advancing haustorium. Smooth

endoplasmic reticulum was seen at the vicinity of the host plasmalemma, which at times encased the haustorium noting the formation of endoplasmic reticulum as a major change in comparison to the situation viewed in uninfected cells. Increase of vesicular activity in the host cytoplasm in the vicinity of the haustorium, along with presence of dense vesicles were also noted at the haustorial surface. At later stages of infection (24 h after inoculation) the space between the haustorium and the extrahaustorial membrane enlarged and formed the matrix region. In more mature (old) haustoria, (36 h after inoculation) the sheath was well developed and composed of material with same electron density as that of the host cell wall. The endoplasmic reticulum which was present at 24-30 h after infection was not present at 36 h. No such "blebs" as reported by Armentrout and Wilson (1969) were viewed by the study of Manocha and Lee (1971). Further, no papillae was formed, and the host cell wall was the only barrier to resist the penetration of the parasite which later becomes a collar. No collar around the haustorial neck of P. virginiana was reported by Armentrout and Wilson (1969), where as its presence around the haustorium of P. virginiana was reported by Manocha and Lee (1971). The disappearance of endoplasmic reticulum after completion of the sheath and electron density of the latter suggested that the sheath was composed of cell wall material.

To further test this hypothesis Manocha and Lee (1972) employing high resolution autoradiography found that $^3\text{H N}$ -

acetyl - glucosamine fed to the host fungi had a preferential distribution of silver grains on the cell wall area. When host fungus C. cucurbitarum was infected with the parasite, and then exposed to $^3\text{H N}$ - acetyl - glucosamine at various time intervals following inoculation, the label was shown to be incorporated into the cell wall at all times, and in the interface area only after sheath development had commenced. These results indicated that the sheath is composed of the same material as the host cell wall. These experiments, however, did not rule out the probability of non-specific binding of the label or metabolism of $^3\text{H N}$ - acetyl - glucosamine and subsequent incorporation of a by product. Further experiments were performed by Manocha and Letourneau (1978) to determine the origin and composition of the sheath, using polyoxin D, a selective inhibitor of chitin synthetase to manipulate chitin synthesis. The results from their investigations showed that polyoxin D, suppressed the label incorporation in the cell wall and sheath zone and resulted in a decrease of electron density. These results suggested that the sheath zone around the mature haustorium contained host cell wall material, i.e., chitin. Prior to investigations of Manocha and Letourneau (1978) molecular interaction of fungal pathogen with its plant host had not been shown in any haustorial parasites. All previous interpretations of the sheath origin have been based on visual observations of electron micrographs, and limited biochemical evidence for any of these views has been provided. Manocha and Letourneau

(1978) further suggest that a possibility exists that earlier development of the sheath is a main factor in the resistance of hosts to ingress and infection by their respective parasites. Autoradiographic experiments support this view and show that there is more incorporation of ^3H leucine in the haustorium of compatible than in incompatible varieties (Manocha, 1975). According to Manocha and Letourneau (1978) the incorporation of the label decreases in the susceptible host according to the age of the infection, and closely coincides with the formation of the haustorial sheath. Manocha and Letourneau (1978) imply that the sheath has a function in resistance rather than functioning as a nutritional sink. They further indicate that the composition of the sheath would not be similar in different host-parasite combinations, and finally conclude that "the rapidity and efficiency of the host cell to secrete wall material should be considered a factor, among others, responsible for its resistance to the haustorial parasites" (Manocha and Letourneau, 1978).

CHAPTER 3MATERIALS AND METHODSI Organisms and Cultural Conditions

Cultures of the parasite, Piptocephalis virginiana, Leadbeater and Mercer were routinely maintained on its susceptible host, Choanephora cucurbitarum (Berk and Rav.) Thaxter. Spore suspensions of host and parasite were obtained under aseptic conditions with 30 ml distilled water added to the respective culture plates, centrifuged at 3500 xg, washed thoroughly with distilled water and filtered through cheese cloth to get rid of any mycelial pieces and finally stored in 100 ml flasks. Standard spore suspensions containing 10^7 spore/ml of the host and parasite determined by the use of Haemocytometer were repeatedly used throughout the germination studies. Inoculations were carried out by mixing the spore suspensions of host and parasite and seeding them on a solid medium consisting of malt extract, 20 g; yeast extract, 2 g; agar, 20 g in 1 litre of distilled water, at PH 6.5, incubated at $23^{\circ} C \pm 1^{\circ} C$, or on a liquid medium of the same composition as above, minus agar. All media were autoclaved at 15-20 P.S.I. at $121^{\circ} C$ for 20 minutes. By growing the parasite, P. virginiana, in continuous darkness on C. cucurbitarum according to the method of Berry and Barnett (1957), a pure population of the parasite's spore was obtained, without contamination of the host spores. The growth of the spores of C. cucurbitarum is inhibited under

continuous darkness, while P. virginiana produces spores normally (Barnett and Lilly, 1955). These spores of P. virginiana were used to inoculate the resistant host Phascolomyces articulatus Boedijn ex Benny and Benjamin, and non-host species Linderina pennisporea Raper and Fennell, by mixing the spores of parasite with spores of host or non-host species and seeding them on the same medium as described above. All inoculations were carried out under complete sterile conditions.

II Light Microscopy

A diluted spore suspension of the parasite was prepared (approximately 15-20 spores/field under immersion oil) for studying the morphology of spore germination in P. virginiana. The spores of the parasite were inoculated on slides coated with the solid medium, incubated at 25° C, mounted in Lactophenol stain and closely examined at different time intervals. Photo micrographs of the spores were obtained at 1, 4, 8, 15 and 17 h after inoculation.

The sequence of events in interaction between the parasite and the host and non-host species were followed under the light microscope to substantiate the results obtained under the electron microscope. This was achieved by inoculating a spore suspension containing 10^7 spore/ml of the parasite and the same spore concentration for each host and non-host species on a glass slide previously coated with the medium. The slides were then incubated for 18 h at 25° C. Observations were made continuously on their general morphology, however at 18 h they were mounted in Lactophenol

and photomicrographed. The time required for the germination of the spores of the parasite, host, and non-host fungi was recorded, along with measurement of the spore size. This was achieved by using an ocular micrometer which was calibrated against a stage micrometer. More than 50 spores were measured for each fungus.

Photomicrographs were obtained using a Leitz-ortho plan microscope under immersion oil (X100), recorded on panatomic X film, developed in Microdol X for 10 minutes at 22^o C.

A study was conducted to obtain information on the percent germination of spores and contact development between the germinating spores of P. virginiana and the hyphae of the host and non-host species, and specifically to examine any significant differences in these parameters, should there be any, when comparing each host-parasite combination.

Spore suspensions of the parasite, host and non-host species were obtained according to the method described earlier. Using 1 ml pippets 2 drops of the parasite's spore suspension was added to a slide previously coated with the solid medium. For each host group, 2 drops of each of the host and non-host spore suspension was added to 3 different slides already containing the parasite's spore suspension. The glass slides were then placed in a glass petri-dish and kept moist by adding a wet filter paper in the bottom of the petri dish and incubated at 25^o C for 18 h. Slides of these cultures were stained with Lactophenol cotton blue at 18 hr. The stain was prepared by mixing 5 ml of 1% cotton blue in 20 ml of Lactophenol. Each slide was then examined under a

Leitz ortho-plan microscope. This experiment was repeated 5 times under similar experimental conditions. In these germination studies the following four categories were considered and analysed from each slide:

- 1) number of germinated spores of the parasite
- 2) number of non-germinated spores of the parasite
- 3) number of swollen spores of the parasite, and
- 4) number of spores of the parasite contacting the host.

Fifty-two different areas per slide were counted, using 3 different slides for each set of host-parasite combination. The data collected from these experiments were tabulated and summarized in tabular form (see Results). The Mann-Whittney U test was considered to be the most appropriate for analyzing the data collected from these germination studies. In case of Linderina pennispora, no parameters for contacts were recorded, since upon examining numerous different locations, it was concluded that no contact is established between this non-host and the parasite. Therefore only three categories were accounted for with L. pennispora.

III Electron Microscopy

For inoculations of the young host cultures, spore suspensions of P. virginiana and of the host or non-host species were seeded on the malt-yeast-extract agar medium. In inoculations of old host cultures, spores of the host species were not used, instead the cultures were first allowed to grow in a petri dish for 4-6 days until the medium was completely covered before they were challenged with the spores

of the parasite.

For electron microscopy the cultures were fixed at definite intervals of 18, 22, 24, 32, 36, 48 h, after inoculation in 3% glutaraldehyde prepared at 0° C in 0.1 M phosphate buffer PH 6.2 for 2½ h. After being thoroughly washed in buffer they were post fixed in 1% OS O₄ prepared in the same buffer for 2-3 h. The fixed mycelia were dehydrated in ethanol and propylene oxide series and embedded in Spurr's epoxy resin (Spurr, 1969). Silver and yellow thin sections were cut on a Reichert Ultramicrotome using glass knives or Dupont diamond knife. These sections were then mounted on copper grids and stained with lead solution (Reynolds, 1963) for 5-8 minutes, and examined in a Philips 300 transmission electron microscope.

CHAPTER 4RESULTS

The results of the present investigation are presented under two separate sections (I) Light microscopy and (II) Electron microscopy.

I Light Microscopy

All light microscope observations were recorded from a series of events that occurred at 18 h post seeding of the mixed spore suspension of Piptocephalis virginiana and host or non-host species on a malt-yeast-agar medium. The sequence of events as observed under the light microscope were similar for both the susceptible Choanephora cucurbitarum and resistant Phascolomyces articulatus hosts when challenged with P. virginiana. However, the non-host species, Linderina pennispora showed no apparent interaction with the parasite.

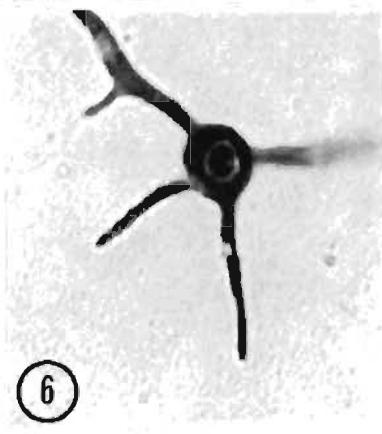
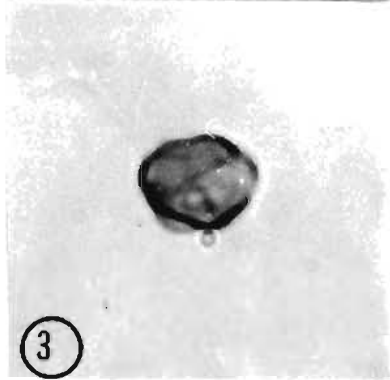
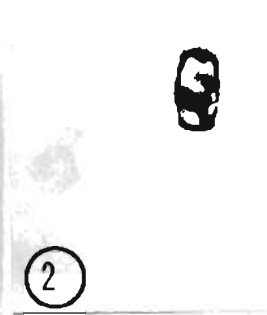
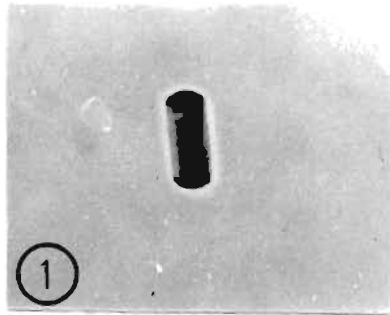
A Morphological Studies(i) Morphology of Germinating Spores of Piptocephalis virginiana

The spores of P. virginiana were smooth and cylindrical with an average size of $6 \times 2.4 \mu$ (Fig. 1). Approximately 4 h after inoculation the spores began to swell slightly (Fig. 2). After 8-10 h, these asexual spores swelled to about two to three times their original size, became globose in shape (Fig. 3) and by 15-17 h the emergence of germ tubes became

Abbreviations

Ap	=	appressorium
Cw	=	cell wall
Ex	=	extrahaustorial membrane
Gt	=	germ tube
H	=	host
Ha	=	haustorium
Ip	=	infection peg
Iw	=	inner wall layer
M	=	mitochondrion
N	=	nucleus
Ow	=	outer cell wall
P	=	parasite
Pa	=	papilla
Pm	=	plasma membrane
S	=	sheath
V	=	vacuole
Ve	=	vesicle

- Figs. 1-6 Morphology of Spore Germination in
P. virginiana.
- Fig. 1 Spore of the parasite 1 h after
inoculation, having a smooth surface
and a cylindrical shape. X 580.
- Fig. 2 Light-microscope photograph of a
slightly swollen spore 4 h past
inoculation. X 580.
- Fig. 3 Globose spore 8 h following
inoculation. X 580.
- Fig. 4 Initial emergence of a germ tube.
X 580.
- Fig. 5 Spore of the parasite with 2 germ tubes,
emerging from opposite ends of the spore,
15 h after inoculation. X 410.
- Fig. 6 Single spore with 4 germ tubes.
X 450.



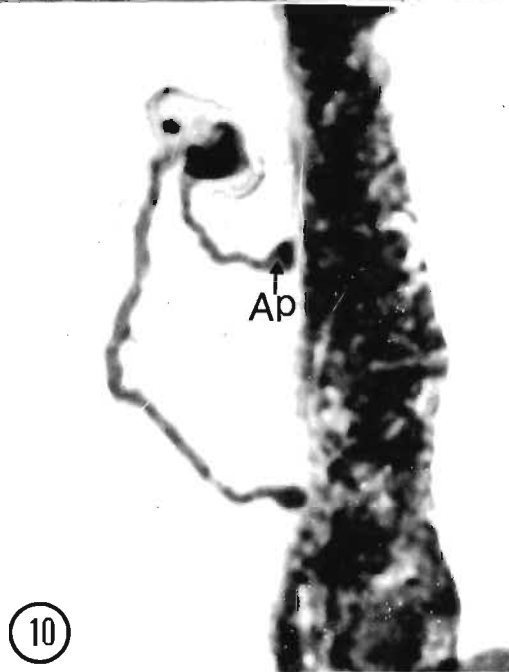
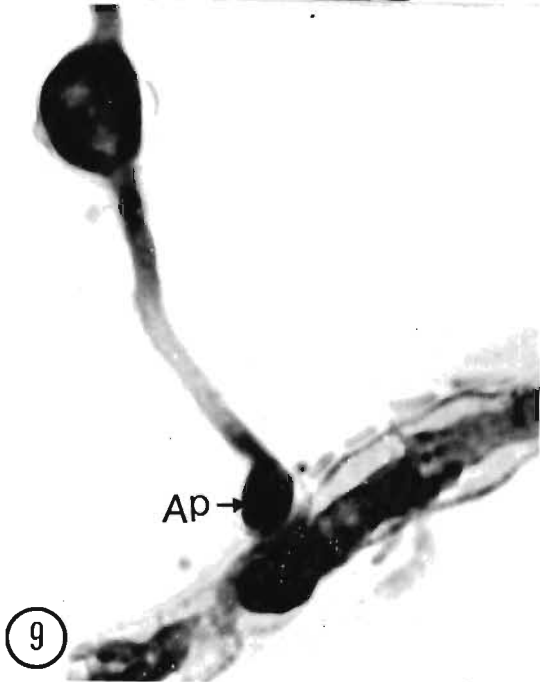
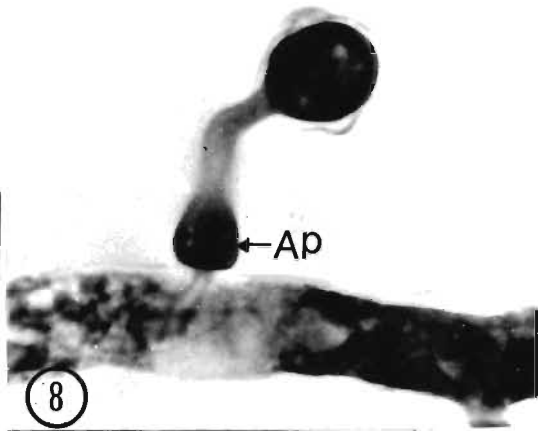
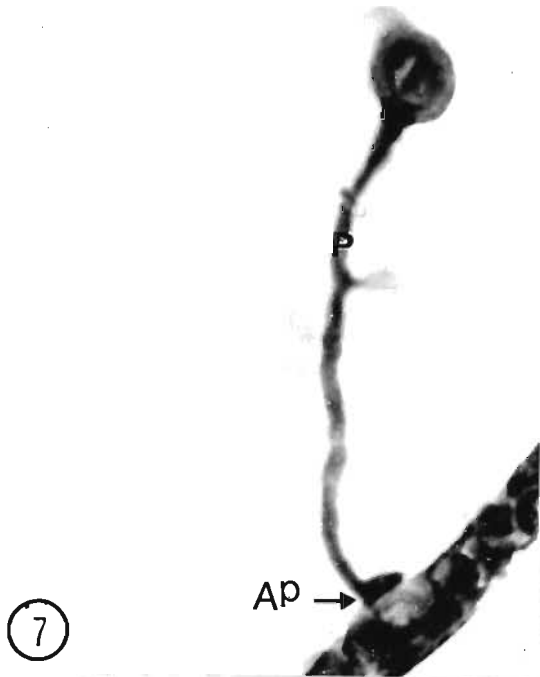
apparent (Fig. 4). Each spore was capable of producing from one to four germ tubes (Figs. 5 and 6), but generally it produced two germ tubes which did advance or branch at random. When the mixed spore suspension of P. virginiana and C. cucurbitarum was inoculated on the same plate containing malt-yeast-extract medium, the spores of the host germinated within 5 h, whereas the first germ tube from the parasite's spore was not observed until approximately 15 h after inoculation.

Mycelia of P. virginiana were normally easily distinguishable from those of both the resistant and susceptible hosts, since the host mycelia were greater in diameter than those of the parasite. The same was applicable in regards to the parasite's spore. As an example, spores of C. cucurbitarum were oval in shape and brown in color with an average size of $15 \times 11 \mu$ as compared to the $6 \times 2.4 \mu$ rod shaped spores of the parasite.

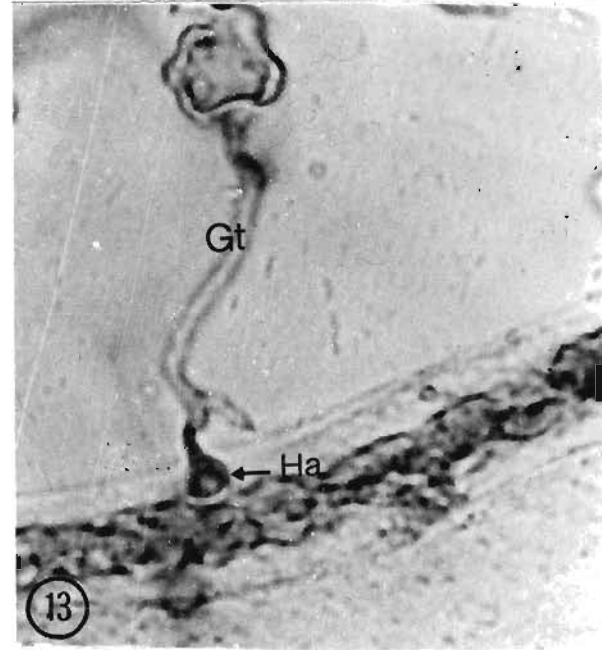
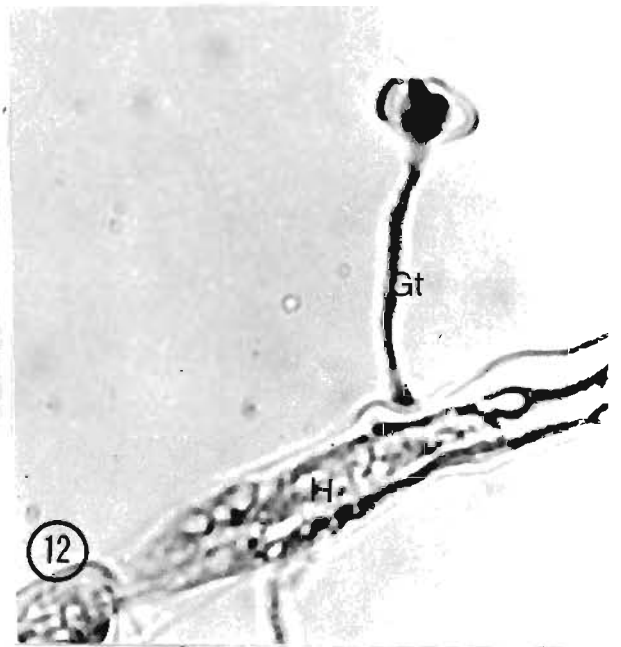
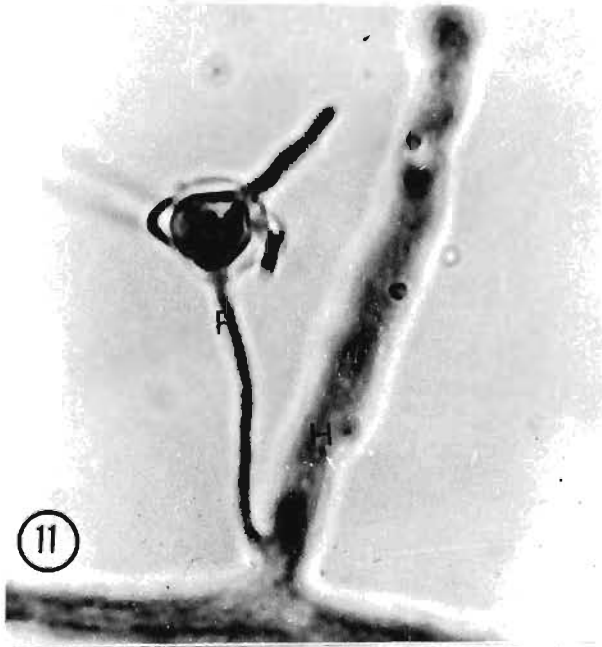
(ii) Interaction Between Piptocephalis virginiana and Choanephora cucurbitarum

Figures 7 to 14 exhibit the interaction between P. virginiana and C. cucurbitarum as observed under the light microscope. The spores of the parasite when inoculated along with the host, usually did put out two germ tubes sometimes maybe one, from the side nearest to the host hyphae (Figs. 7, 8, 10, 11, 12 and 13). The germ tube then grew directly towards the host hyphae making contact and penetrating the hyphae. Frequently the spores were observed to grow towards

- Figs. 7-14 Light-microscope photographs of the interaction between P. virginiana and C. cucurbitarum.
- Fig. 7 A single germ tube growing directly towards the host hypha, with a slight swelling (appressorium) at the tip of the germ tube. X 590.
- Fig. 8 Well developed appressorium leaning on the host cell wall. X 580.
- Fig. 9 Depression of host cell wall by the appressorium. X 580.
- Fig. 10 Double infection of the host cell. X 700.



- Fig. 11 Penetration of the host hypha by the germ tube of the parasite. X 650.
- Fig. 12 Host cell wall pushed inwards by the appressorium. X 600.
- Fig. 13 Haustorium formed in the host hypha. X 750.
- Fig. 14 Penetration of the host hyphal tip by the germ tube of the parasite. X 750.



the host hyphae, especially in the direction of the thin-walled emergent hyphal tip which is highly susceptible to infection (Fig. 14).

As the germ tube approached the host cell, the tip of the germ tube swelled over the surface of the host cell wall to form an appressorium (Figs. 7, 8 and 9). Once the appressorium was formed the host cell wall seemed to become depressed by the pressure exerted on it by the appressorium (Figs. 9 and 12). The well developed appressorium can be seen in Figs. 8 and 9. Formation of a small appressorium like swelling by the parasite at the point of contact with this susceptible host was followed by penetration by an infection peg and the development of a haustorium (Figs. 13 and 14). Germination, penetration and formation of haustoria (by P. virginiana) required as little as 18-20 h, when the parasite and C. cucurbitarum were in close proximity. Very rarely a double infection of the susceptible host hyphae was observed (Fig. 10), although this was a common observance with the resistant host.

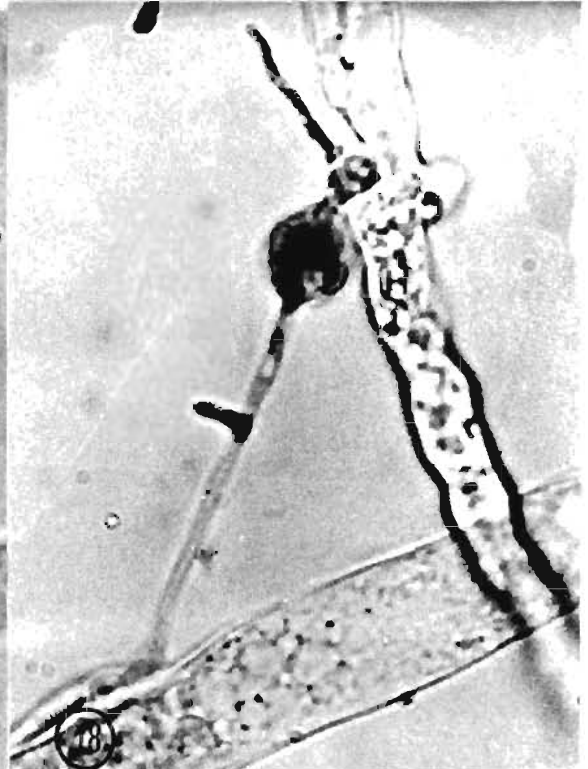
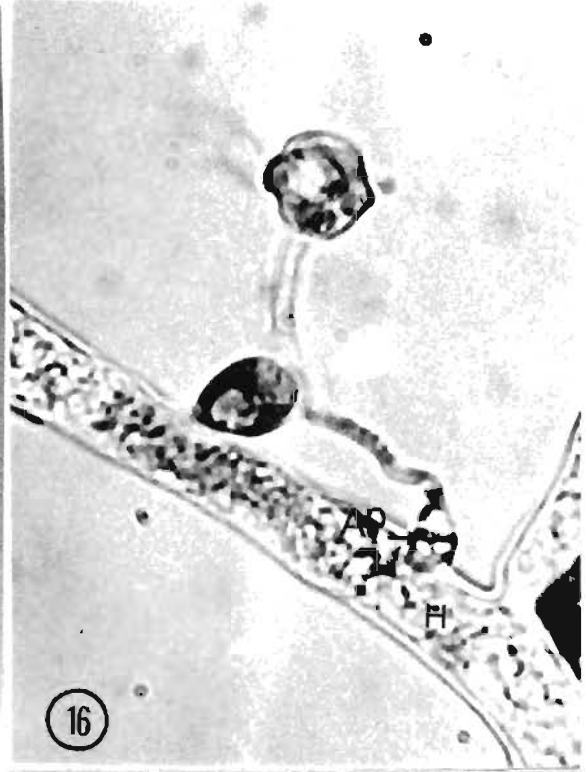
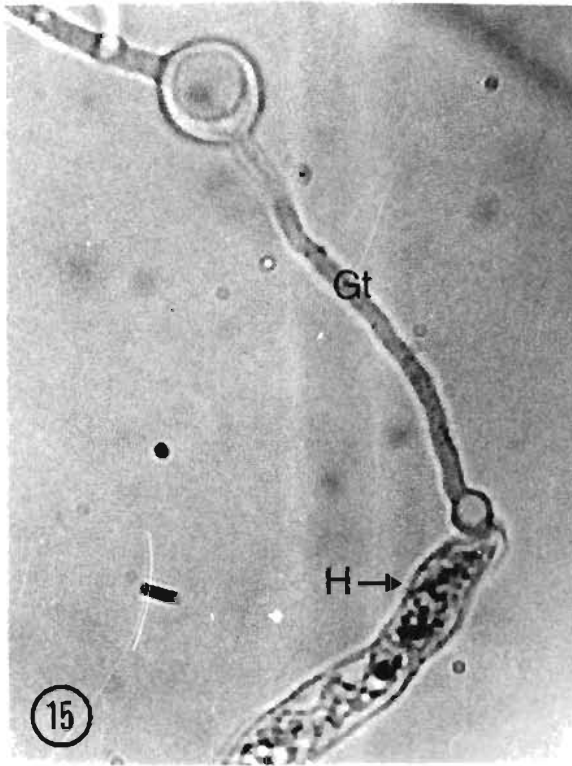
Further development of the parasite after the initial penetration of the host hyphae has been described in detail by Lee (1971). The first sporophores of the parasite were apparent on 3 day old cultures. P. virginiana produced clusters of spore chains on dichotomously branched sporophores when grown with the susceptible host C. cucurbitarum Manocha and Lee (1971). The sporophores of the parasite are tan colored against the background of the host. Subsequent growth of the parasite on this highly susceptible host was

rapid, with maximum growth occurring within 5 days, actively parasitizing the host and covering the surface of the petri dish.

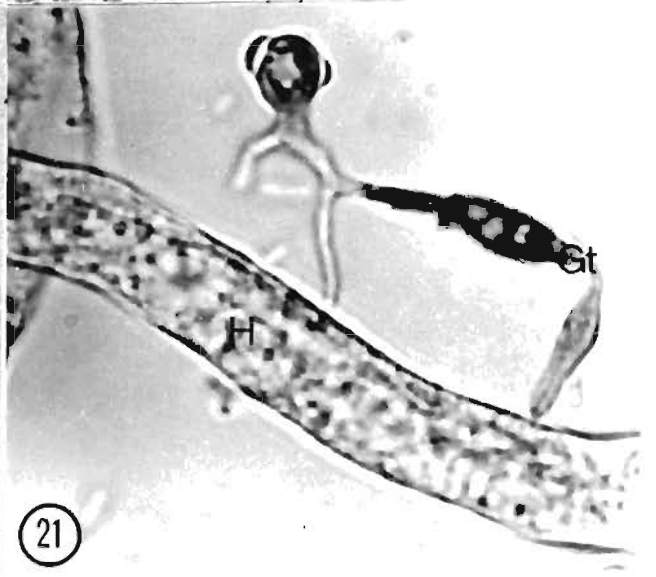
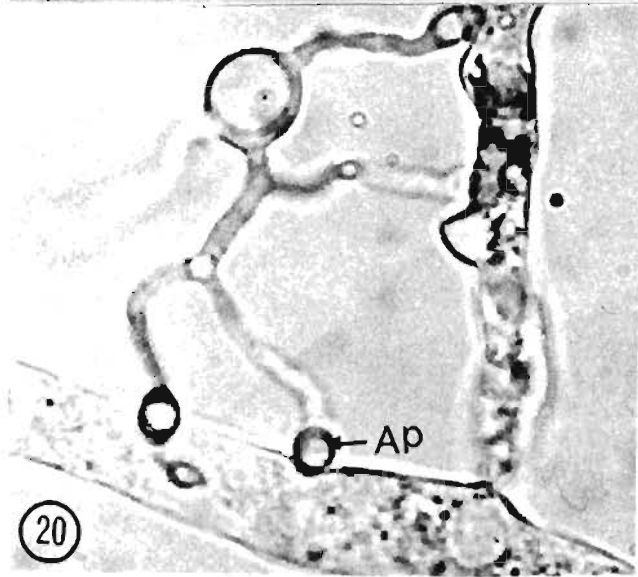
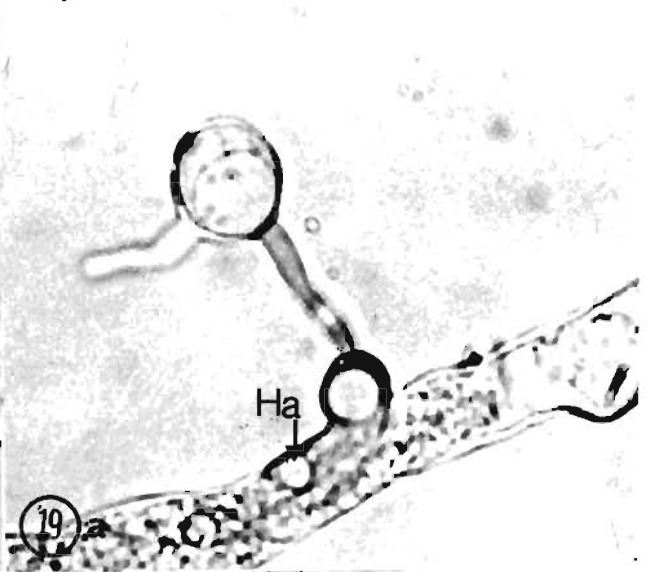
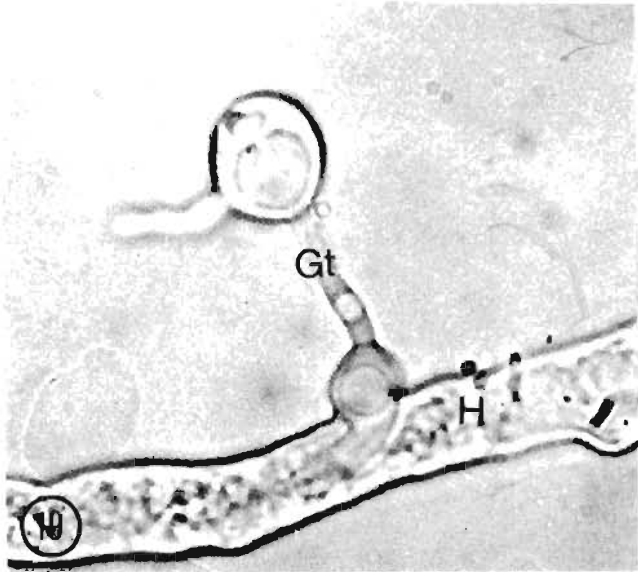
(iii) Interaction Between Piptocephalis virginiana And
Phascolomyces articulatus

Different stages in the interaction between P. virginiana and the resistant host P. articulatus closely resemble those of the susceptible host C. cucurbitarum when viewed under the light microscope (Figs. 15-24). When a mixed spore suspension of the parasite and P. articulatus was inoculated on malt-yeast-extract medium, it was observed that spores of P. virginiana did not germinate prior to 15 h, whereas the sporangioles of P. articulatus swelled and developed germ tubes within 5 h. The growing hyphal tip of the host seemed to be attacked more frequently than other sites in the host hyphae (Figs. 15 and 22). In most instances, the germ tube contacting the resistant host hyphae of P. articulatus was produced by the side of the parasite's spore nearest to the host hyphae, as was the case with the susceptible host C. cucurbitarum (Figs. 17, 19, 19a, 20 and 21). The germ tube then grew in the direction of the host hyphae contacting and penetrating the hyphae (Figs. 16 and 19). The germ tubes of the parasite have been observed to develop branches when in contact with the hyphae of P. articulatus (Figs. 22, 23 and 24). This phenomenon was not observed in the interaction between P. virginiana and the susceptible host C. cucurbitarum.

- Figs. 15-24 Light-microscope photographs of the interaction between P. virginiana and P. articulatus.
- Fig. 15 Growth of the parasite's germ tube directly towards the hyphal tip of the host. X 550.
- Fig. 16 Formation of the appressorium on the host hypha. X 600.
- Fig. 17 A germ tube contacting the host hypha put out from the side closest to the host hypha. X 600.
- Fig. 18 An infection peg inside the host hypha. X 750.



- Fig. 19 Infection peg inside the host cell
X 600.
- Fig. 19 (a) The same as Fig. 19, but at different
focus. Note the haustorium in the host
cell. X 600.
- Fig. 20 A single spore with 4 germ tubes
attacking the host hyphae, each
possessing a well developed appressorium.
X 600.
- Fig. 21 Double infection of the resistant host
hypha, rarely observed in the susceptible
interaction. X 650.

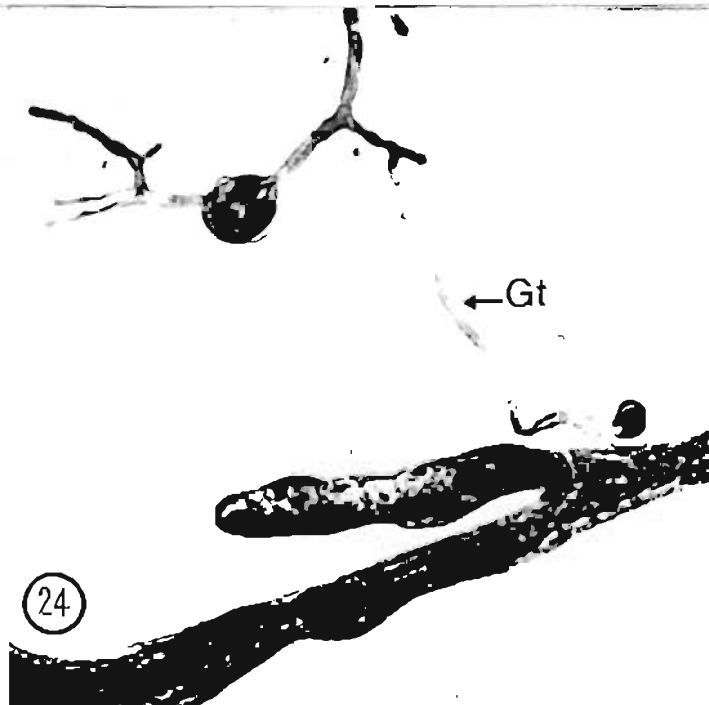
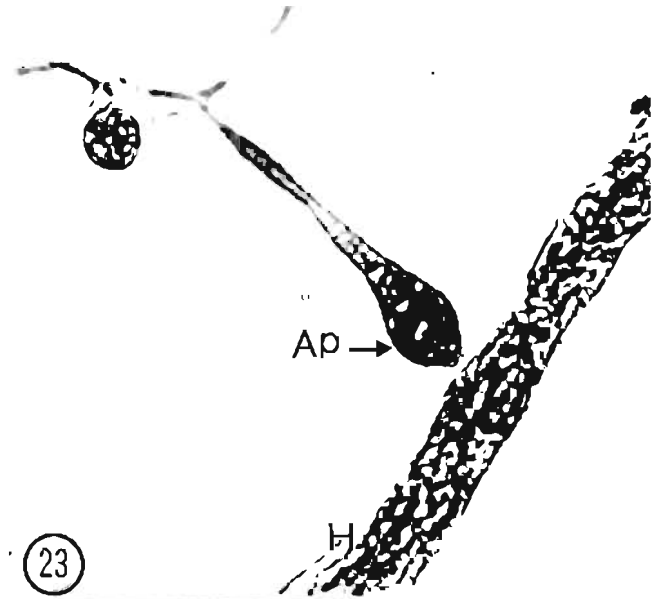
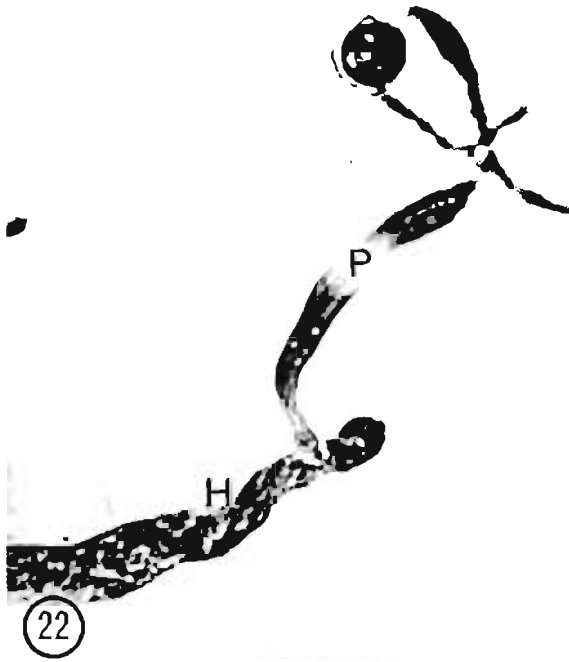


The tip of the germ tube swelled, forming an appressorium which attached tightly to the surface of the host hyphae (Figs. 15, 16 and 24). An infection peg was formed, and the host cell was penetrated by this infection peg which developed into a haustorium (Figs. 17 and 18). Figs. 19 and 19 (a) are the same, but taken at different foci, from which the haustorium located within the host cell can be observed. The interaction between this resistant host and P. virginiana required 18-20 h, which is similar to that of the susceptible host C. cucurbitarum.

Great variation was observed in the general appearance (morphology) of the parasite's germ tube, in terms of width, length and shape when infecting the resistant host P. articulatus (compare Figs. 15, 19, 21 and 24). Another point of interest was the observation, that the parasite made multiple penetration attempts of the resistant host hyphae (Fig. 20). Even the double infection commonly observed in P. articulatus (Fig. 21) was rarely seen in the case of C. cucurbitarum (Fig. 10). The susceptible host was never observed to be attacked by 4 germ tubes of the parasite whereas this phenomenon was occasionally seen in the resistant host.

The parasite's growth on the resistant host was very slow. Even though the parasite attacked the hyphae of P. articulatus, further growth was apparently curtailed and the hyphae of P. articulatus continued to elongate and rapidly out grew the parasite. The parasite was never observed to produce sporophores. This situation contrasts markedly with the compatible host-parasite association between P. virginiana

- Fig. 22 Germ tube attacking the hyphal tip
of the host. X 450.
- Fig. 23 Appressorium contacting the host
hypha . X 420.
- Fig. 24 Highly branched germ tube, contacting
the host hypha . with an appressorium.
Note the difference in morphology of
germ tubes between Figs. 22-24 and
15-21. X 400.



and C. cucurbitarum where the host was actively parasitized in plate culture and normally over grown. However P. virginiana caused no apparent damage to either the susceptible or resistant host fungi.

(iv) Interaction Between Piptocephalis virginiana And Linderina pennispora

Figures 25-28 demonstrate the interaction between P. virginiana when grown with the non-host L. pennispora. Upon inoculation of a mixed spore suspension of P. virginiana and the non-host, L. pennispora, on a malt-yeast extract agar medium, the parasite's spores germinated within 15-17 h, and sporangiales of L. pennispora swelled and developed germ tubes within 6 h. However the non-host fungus exhibited no interaction with the parasite.

The spores of the parasite germinated in the presence of this non-host species, but the germ tubes did not exhibit directional growth towards the hyphae of L. pennispora and their growth appeared to be random in direction. Moreover, no contact or penetration of this non-host fungus was observed. In instances where the germ tube of the parasite was seen to grow towards the hyphae of L. pennispora the germ tube bypassed the ^{non-}host hyphae, appearing as if the parasite failed to even recognize the presence of the hyphae of this non-host (Figs. 25-28). Occasionally, what appeared to be a parasite's contact with the non-host hyphae was found upon manipulation of the microscopic focus, to be merely a visual mistake rather than an attachment. These conclusions were drawn upon

Figs. 25-28 Light-microscope photographs of interaction between P. virginiana and the non-host L. pennispora.

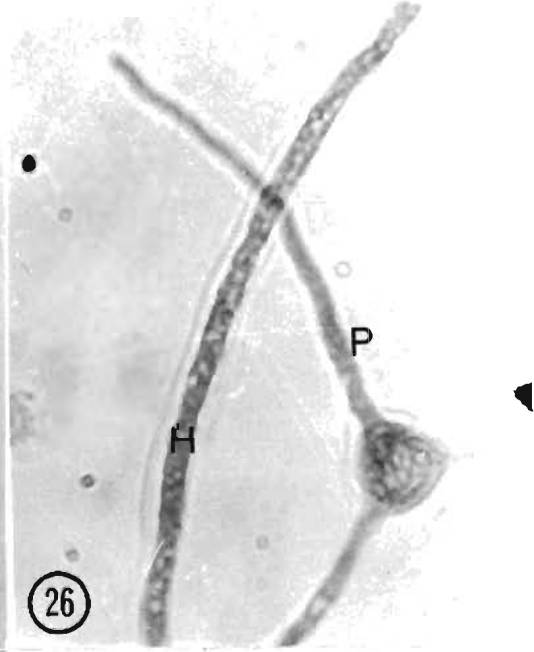
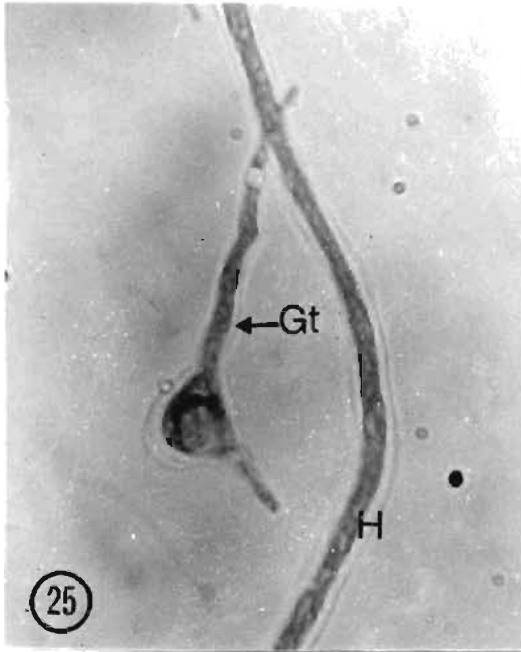
The parasite's germ tube germinates in presence of this non-host. However the germ tubes show no attachment or contact of this non-host, and by-pass the hyphae of L. pennispora even when they are in close proximity.

Fig. 25 X 470.

Fig. 26 X 500.

Fig. 27 X 550.

Fig. 28 X 470.



examination of at least 100 different sites.

Due to the limitations imposed by light microscopy it was decided to investigate this problem further under the electron microscope to reveal the details of fine structure and sequence of events occurring during interaction of each host and non-host species when challenged with P. virginiana.

B Germination Studies

The data in Table I show the percent germination, contact formation and penetration of P. virginiana spores in the presence of host and non-host species. It is evident that there was no marked difference in percent germination of P. virginiana spores and in the penetration of the susceptible and resistant hosts. However, the percent germination of P. virginiana was lower in the presence of a non-host species L. pennispora and there was no penetration by the parasite of this non-host. Statistical analysis of the data using Mann-Whitney U test showed no significant difference when the ($P \leq 0.05$) values of percent germination of the parasite's spore in the presence of host and non-host species were compared. Similarly no significant difference was obtained when comparing the values of percent penetration of the parasite's spores of the resistant and susceptible hosts.

II Electron Microscopy

- (i) Fine Structure of the Parasite, Piptocephalis virginiana
Cytoplasm of P. virginiana was filled with ribosomes,

TABLE I

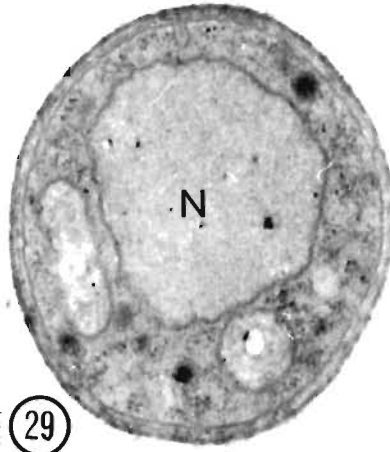
Percentage germination and penetration of spores of
P. virginiana inoculated with spores of C. cucurbitarum,
P. articulatus and L. pennispora.

	<u>C. cucurbitarum</u>	<u>P. articulatus</u>	<u>L. pennispora</u>
Total number of <u>spores</u>	2237	1599	1925
Spores <u>germinated</u>	1749	1261	930
Spores <u>penetrated</u>	889	567	---
Spores <u>swollen</u>	276	183	372
Spores <u>not germinated</u>	212	155	623
% spores germinated	78.20	78.86	47.93
% spores penetrated	39.74	35.45	---

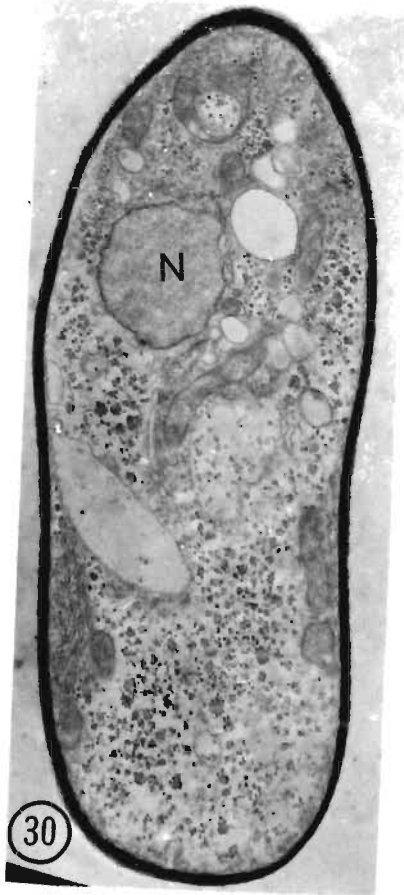
Figs. 29-30 Fine structure of P. virginiana.

Fig. 29 Cross section of a germ tube. Note homogeneous, central nucleus, in a cytoplasm packed with ribosomes, and surrounded by a thin smooth cell wall. X 35000.

Fig. 30 Oblique-longitudinal section of the parasite. X 22000.



29



30

mitochondria, endoplasmic reticulum and small vacuoles as can be observed from Figs. 29 and 30. The cytoplasm delimited by the plasmalemma was enclosed in a thin single layer of cell wall. The nucleus with characteristic double membrane had homogeneous nucleoplasm and occupied the major portion of the cell (Fig. 29). The nucleus demonstrated the double membrane structure normally interrupted by pores (not shown in these figures). The germ tube of P. virginiana was approximately one third in width of each of the host and non-host hyphae, but generally the organelles of the parasite were similar to those of the host cell except for their smaller size

(ii) Fine Structure of Choanephora cucurbitarum

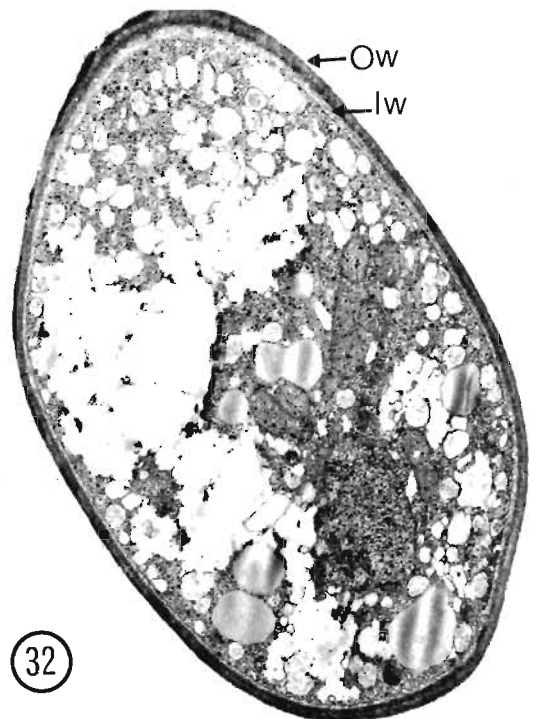
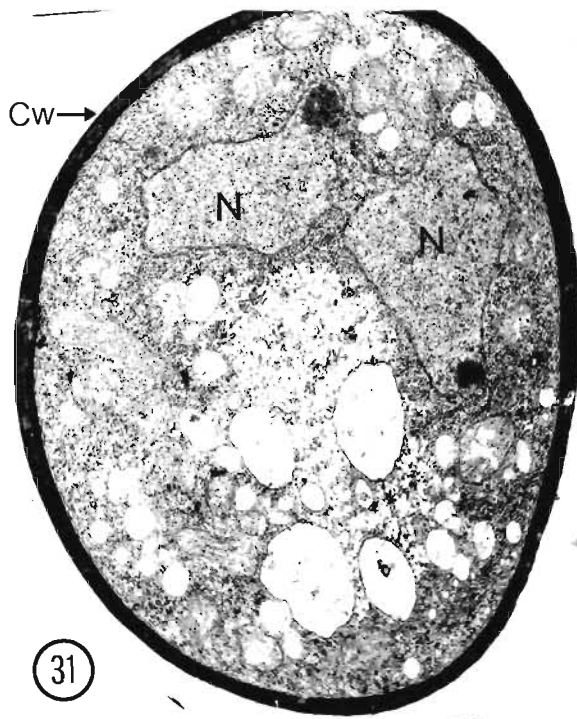
A smooth single layered cell wall surrounded the cytoplasm with the plasmalemma closely appressed to the cell wall, in a 24 h old cell of C. cucurbitarum. The cytoplasm contained numerous ribosomes, mitochondria few cisternae of endoplasmic reticulum and vacuoles. Two well defined nuclei each enclosed in a double membrane and distinct nucleoli, were present (Fig. 31).

In a 4-5 day old hyphae, the cell wall was distinctly differentiated into 2 layers, with different degrees of electron density, characteristic of the old hyphae of C. cucurbitarum (Fig. 32). Numerous vesicles were observed in the cytoplasm of an old hyphae as compared to the young cell.

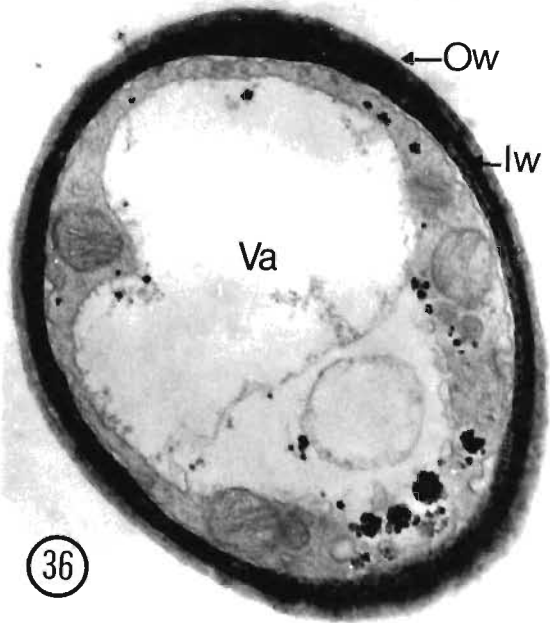
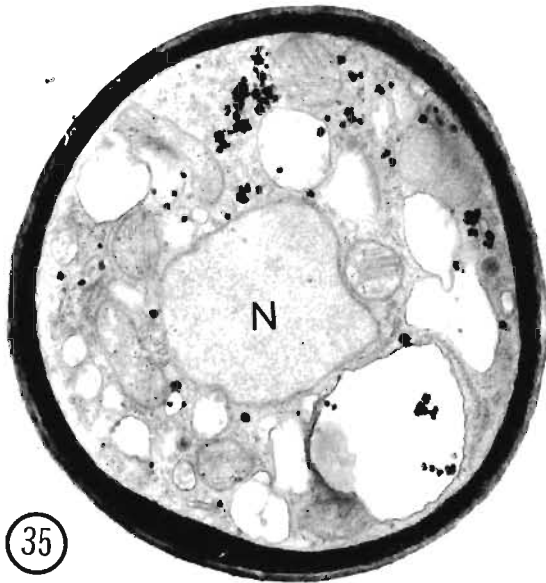
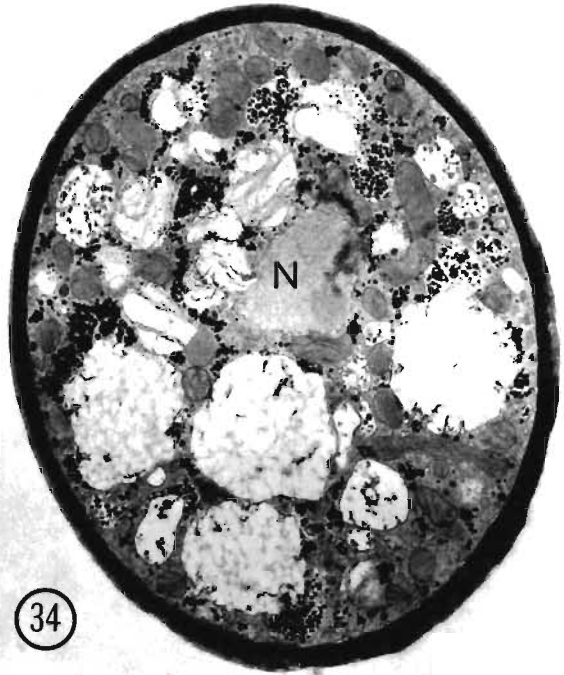
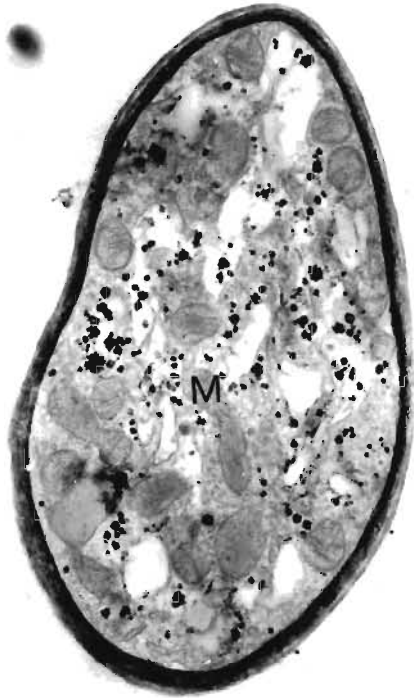
Figs. 31-32 Fine structure of C. cucurbitarum.

Fig. 31 Thin section of a 24 h old cell,
showing single layered cell wall with
2 distinct nuclei and nucleoli.
X 18000.

Fig. 32 26 h old cell, exhibiting the double
layered cell wall with different degrees
of electron density. X 15000.



- Figs. 33-36 Fine structure of P. articulatus.
- Figs. 33,34 20 h old cells of P. articulatus.
Note the double layered thick cell
wall. X 19000 and 195000 respectively.
- Fig. 35 Thin section of a 36 h old cell showing
a central nucleus. X 21000.
- Fig. 36 42 h old cell exhibiting extensive
vacuolation. Note the thickness of the
cell wall as compared to the other fungi.
X 22000.



(iii) Fine Structure of Phascolomyces articulatus

Cells of P. articulatus exhibited one common characteristic feature, that being the smooth, thick double-layered cell wall, with different electron density. The 20 h old cells of P. articulatus (Figs. 33 and 34) showed a distinct double layered cell wall as compared to the thin single layer present in C. cucurbitarum. The cytoplasm of these cells was filled with numerous mitochondria with double membranes and well developed cristae, ribosomes, cisternae of endoplasmic reticulum, vesicles and a well defined nucleus with the typical double membrane, containing a homogenous nucleoplasm. In a 36 h old cell (Fig. 35) the nucleus was located in the centre, with the mitochondria, endoplasmic reticulum, and few vacuoles surrounding it. At 42 h after inoculation, extensive vacuolation was noticed in the cells. The cell wall was double layered and very thick (Fig. 36). The thickness of cell walls in P. articulatus was more pronounced as compared to the other fungi described previously. Cells of P. articulatus contained storage material within their cytoplasm, however no attempts were made to identify them.

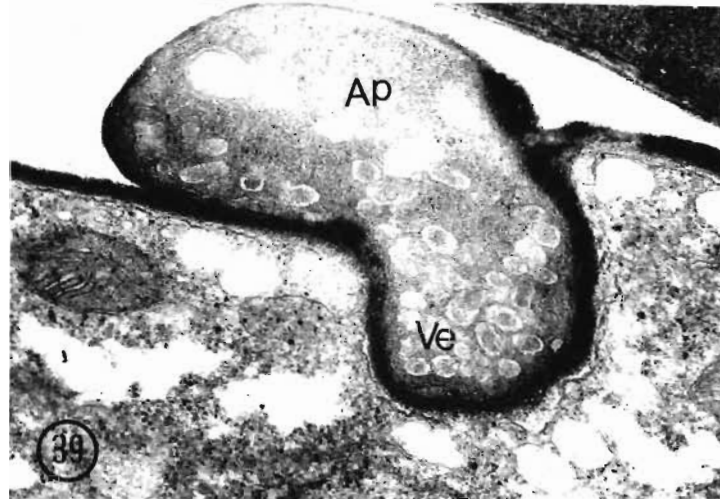
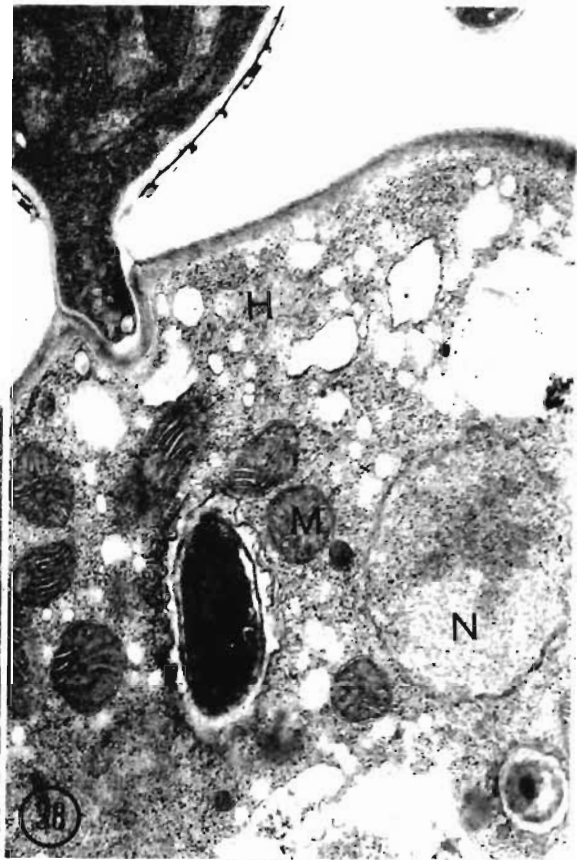
(iv) Interaction Between Piptocephalis virginiana and Young Choanephora cucurbitarum.

Different stages of infection of P. virginiana on young C. cucurbitarum are shown in Figs. 37-45. The germ tube of P. virginiana grew directly towards the susceptible host C. cucurbitarum and established contact with the host cell wall, pushing the host cell wall inwards (Figs. 37 and 38). As the

Figs. 37-45 Electromicrographs of interaction between P. virginiana and young C. cucurbitarum.

Figs. 37-38 Germ tubes in close contact with the thin single-layered host cell wall which has been pushed inwards. Note accumulation of host mitochondria near the infection hypha. X 30000.

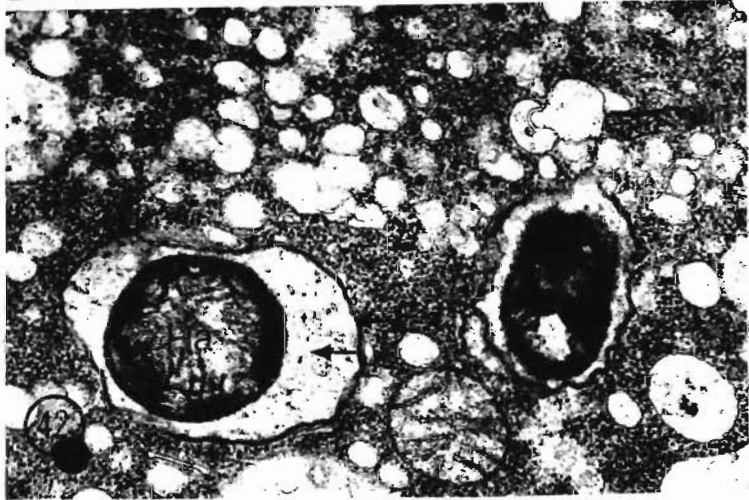
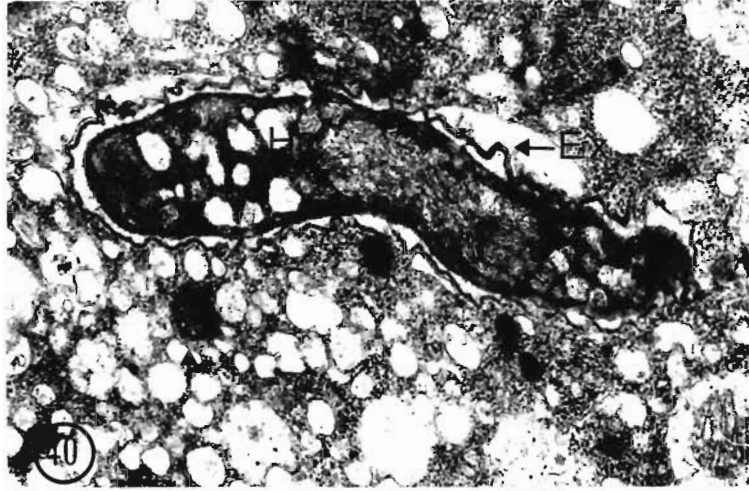
Fig. 39 An infection peg, with numerous vesicles. Note the invaginated plasma membrane. X 35000.



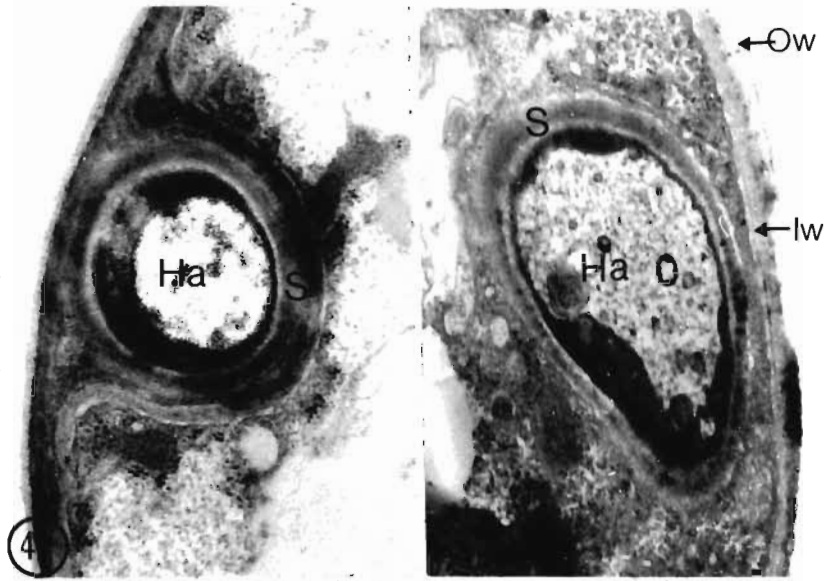
parasite's germ tube approached the host cell, the tip of the germ tube swelled over the surface of the host cell wall to form an appressorium (Fig. 39). An infection peg then formed which penetrated the host cell, invaginating the cell wall and forming a dome-shaped protuberance. The advancing infection peg was attached tightly to the host wall, making the distinction between the host cell wall and the wall of the infection peg difficult. The cell wall of the young (18-24 h old) C. cucurbitarum was thin and composed of a single layer (Figs. 37, 38, 39). The plasma membrane of the host invaginated along the involuted cell walls and did not seem to rupture (Fig. 39). Host response to the parasite in the form of a papilla was absent. The depression of the cell wall of a susceptible host by the penetration peg is indicative of mechanical pressure. Penetration of P. virginiana in this case appeared to be mechanical due to inward push and rupture of the thin single layered host cell wall. Moreover, changes in the structure of the host cell wall normally accompanying enzymatic penetration were not observed. This could not be established with certainty due to lack of enzymatic tests in this study.

The appressorium and infection peg contained avacuolate cytoplasm packed with ribosomes (Figs. 37 and 38) along with numerous characteristic apical vesicles, observed in the infection peg (Fig. 39). Mitochondria of the host were observed to accumulate in the immediate vicinity of the infection peg with associated nucleus below them (Figs. 37 and 38).

- Fig. 40 Young haustorium 18-20 h following inoculation, enclosed in an extrahaustorial membrane. Note the vesicular activity in the vicinity of the haustorium (arrow) and the absence of sheath development. X 29000.
- Fig. 41 A haustorium 22 h after inoculation, showing vesicles between the haustorial wall and the extrahaustorial membrane (arrow). X 32000.
- Fig. 42 Thin section of host-parasite interface 24 h following inoculation, showing the appearance of a distinct zone with vesicular activity (arrow). X 33000.



- Fig. 43 36 h old haustorium completely encased
by the sheath material. X 21000.
- Fig. 44 Haustorium encased in a sheath, with
similar electron-density as that of
the inner layer of host cell wall.
X 30000.
- Fig. 45 Various haustoria surrounded by the
sheath material. X 37500.



Upon rupturing of the host cell wall, the infection peg formed a haustorium within the host cell. The young haustorium (18-20 h after infection) enclosed in its own cell wall was tightly surrounded by the host plasma membrane which was highly invaginated and hereafter referred to as the extra-haustorial membrane (Fig. 40). There was little space between the haustorium cell wall and the extrahaustorial membrane during early stages of infection and it lacked the electron-opacity commonly observed in the resistant interactions. Vesicular activity in the immediate vicinity of the haustorium along with few vacuoles was noticed in the host cytoplasm (Fig. 40). At 22 h after inoculation, the extrahaustorial membrane began separating from the haustorium, along with a concomitant increase of vesicles between the haustorial wall and the extra-haustorial membrane (Fig. 41). When infection reached 24 h, the extrahaustorial membrane completely separated from the haustorial cell wall resulting in the formation of a distinct electron transparent zone (Fig. 42). Extensive vesicular activity was observed in the haustorial matrix region along with vesicles and mitochondria in the host cytoplasm. Cytoplasm of the haustoria contained ribosomes, vesicles, enclosed in thin layered cell wall. At 20-32 h following inoculation, the enlarged interface zone began to show an accumulation of electron-dense material. This matrix is referred to as the "haustorial sheath." Finally by 36 h the sheath was fully developed showing similar electron density as that of the host cell wall. The sheath development coincided with the host

cell wall which differentiated from one layer to two layers at old hyphal stage (Fig. 45). The sheath completely enclosed the haustoria, isolating these structure from the host cytoplasm (Figs. 43 and 44). No signs of sheath formation were observed at earlier stages of haustorium development (compare Figs. 43-45 with Figs. 40-42).

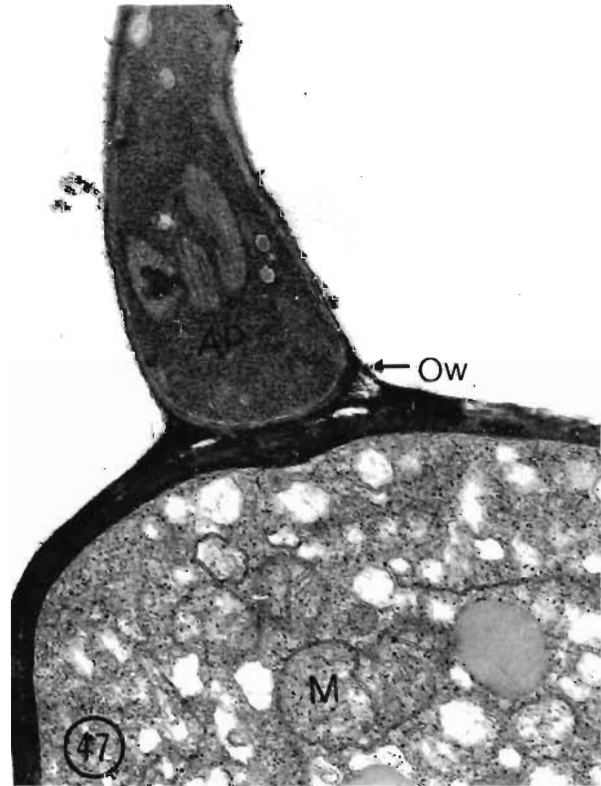
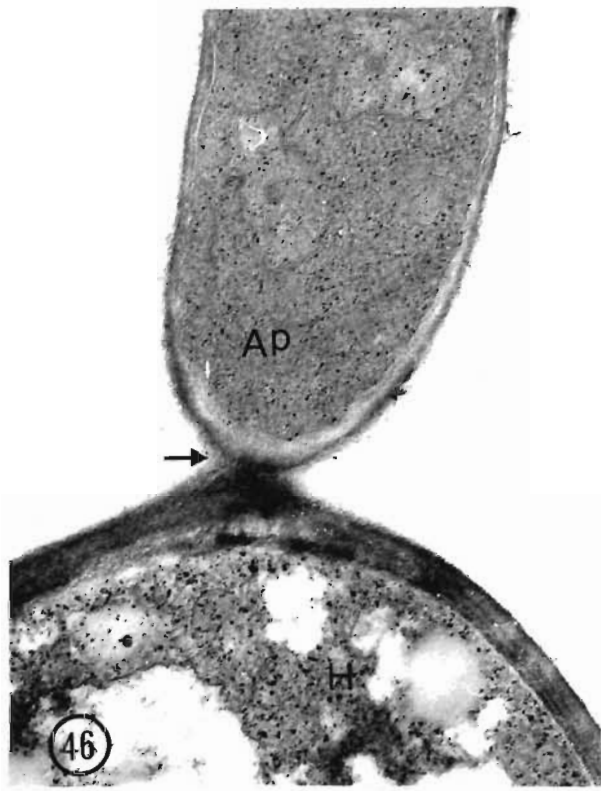
(v) Interaction Between Piptocephalis virginiana And Old Choanephora cucurbitarum

The sequence of events that took place when a 4-5 day old mycelium of C. cucurbitarum was challenged by a freshly prepared spore suspension of P. virginiana are represented by Figs. 46-53. The samples for electron microscopy were obtained at 18-24 h after inoculation.

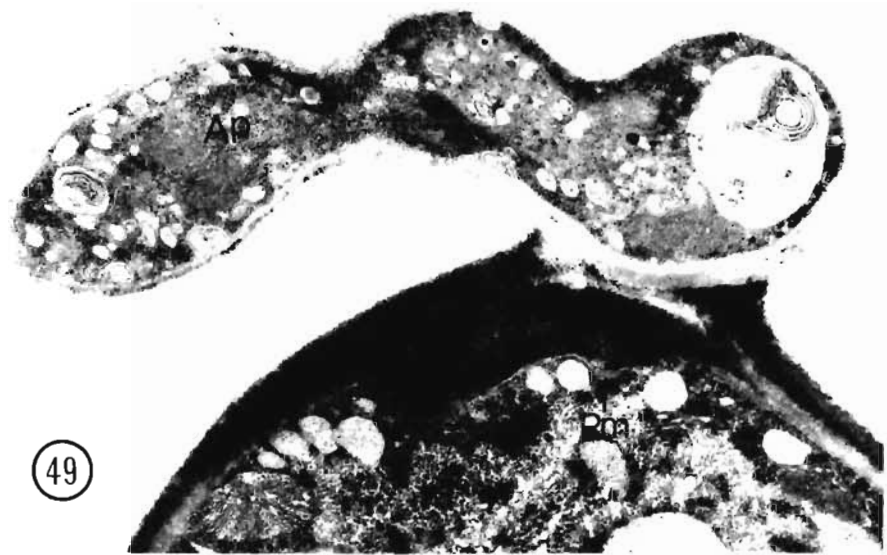
The germ tube of the parasite established a close contact with the host cell through an electron-opaque substance which probably served as a cementing material (Fig. 46). There was no evidence of dissolution or changes in the host cell wall, which was thick and easily discernible as two distinct layers (Fig. 46). The cell wall of the parasite was much thinner than the host cell wall, and its outer layer seemed to merge with the center part of the host cell wall (Figs. 47 and 48). The cytoplasm of the parasite's germ tube was avacuolate, packed with ribosomes and encased tightly by its plasma membrane, a situation comparable to that observed with the young host (Figs. 46 and 47).

Having established contact with the host cell wall the

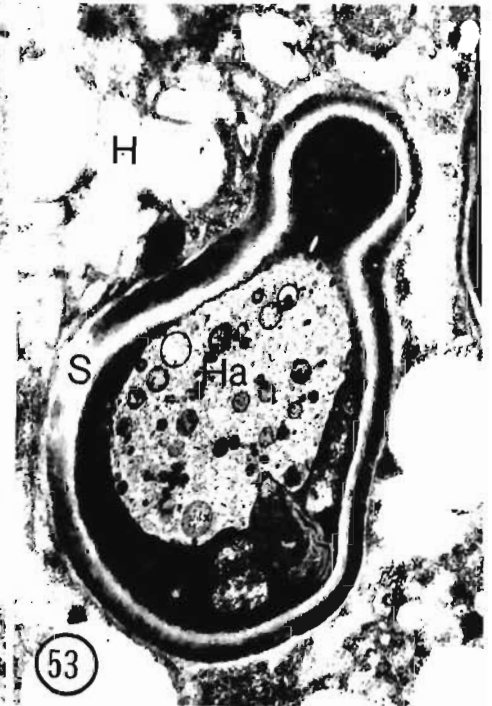
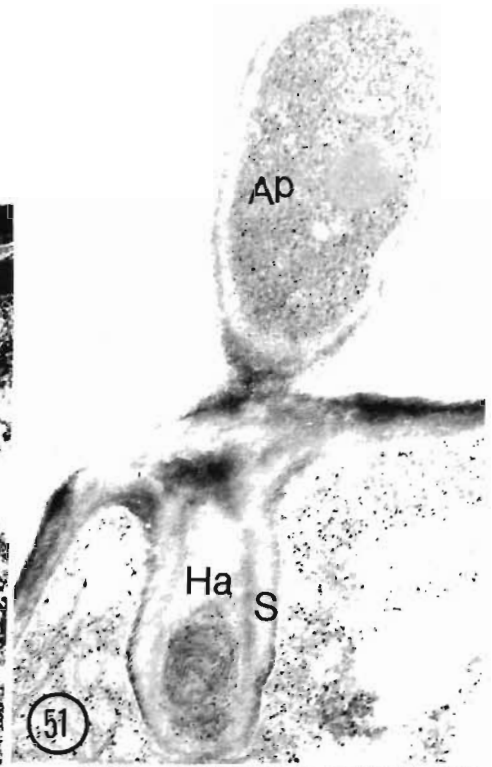
- Figs. 46-53 Electron micrographs of interaction between P. virginiana and old C. cucurbitarum.
- Fig. 46 An initial contact between the host and the parasite. Note the presence of a "cementing" material. X 33000.
- Fig. 47 Initial stage of penetration by the parasite. The outer layer of host cell is dissolved at the point of contact, and appears to partially encase the lower part of the infection peg. X 33000.



Figs. 48-49 Stages in penetration of the outer layer and the development of thickening on the inner layer of the host cell wall. Note the involuted plasma membrane and double-layered host cell wall. X 35000.



- Fig. 50-51 Penetration of the host cell and development of the haustorial peg. Note the presence of electron-dense material around the haustorial peg. X 28000 and 33000 respectively.
- Fig. 52-53 Haustoria of P. virginiana enclosed in an electron dense sheath. In Fig. 52, note the continuity of the inner layer of host cell wall with the sheath material. X 32000 and 28000.



parasite seemed to have dissolved the outer layer of the host cell wall and made contact with the inner layer (Fig. 47). The ruptured outer layer of the host cell wall appeared partially to encase the lower part of the penetrating hypha of the parasite (Figs. 47 and 48). A well developed wall reaction to penetration was noticed, manifested by local thickening of the host wall around the penetration apparatus. The inner layer of the host cell wall became thick in response to the advancing parasite (Figs. 47 and 48), and eventually developed into a small papilla (Figs. 49 and 51). Penetration of the thick papilla by the parasite was not observed. However in few instances where the parasite managed to penetrate the inner layer of the host cell wall and papilla, it failed to establish direct contact with the host protoplast due to the presence of electron-opaque material in the sheath zone. The host plasma membrane was intact and only invaginated at all times. Fig. 49 shows convolutions in this membrane.

The young haustoria (20 h) were enclosed in a material of the same electron-opacity as that of the host cell wall, namely the sheath material (Figs. 50-53). The sheath appeared to be continuous with the inner layer of the host cell wall as observed by Figs. 50-52. The presence of the sheath material surrounding the young haustoria at 18-20 h post inoculation was in contrast to the situation of the haustoria of the same age in the young host where the presence of the electron-opaque material was absent in the sheath zone until later stages of infection (Figs. 40-42). Due to the presence of the thick sheath even at early stages of infection, the parasite at no

time was in direct contact with the protoplast of the old C. cucurbitarum.

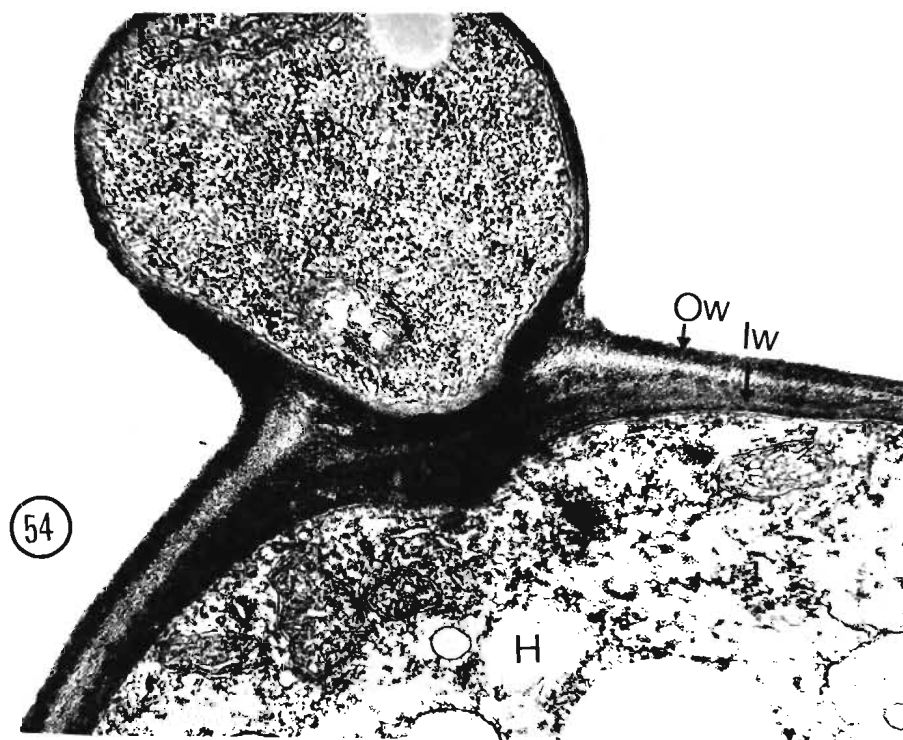
(vi) Interaction Between Piptocephalis virginiana And
Young Phascolomyces articulatus

Upon observation of various thin sections, it was concluded that the interaction between P. virginiana and the resistant host, P. articulatus, illustrated sequence of events similar to those described for P. virginiana and old C. cucurbitarum. Samples were obtained 18-22 h after seeding spore suspensions of host and parasite on malt-yeast-extract-agar medium.

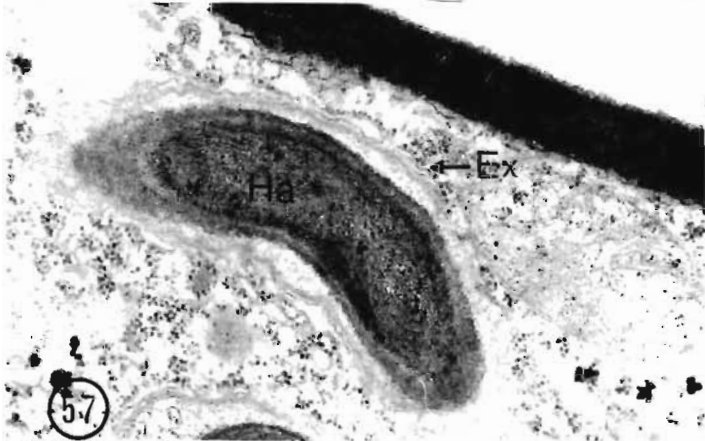
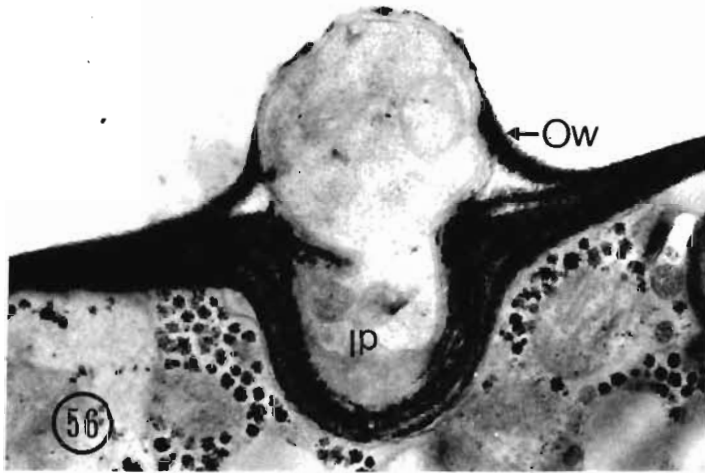
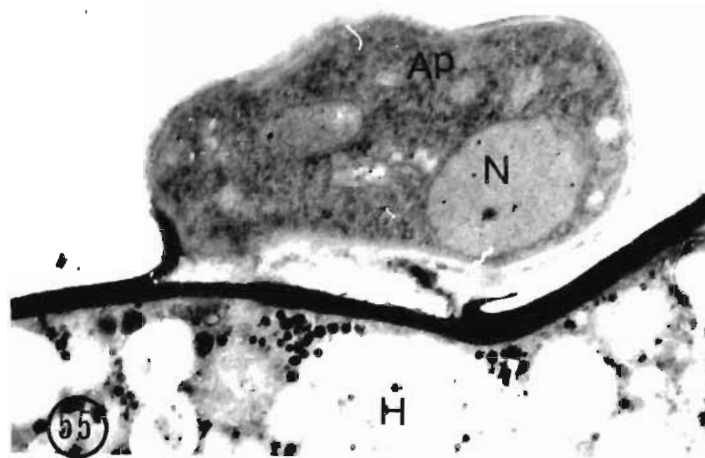
The appressorium of the parasite attached tightly to the host cell, forming an infection peg, which dissolved the outer layer of the host cell wall (Fig. 54). The outer layer seemed to partially encase the invading infection hypha (Figs. 55 and 56). The inner layer of the host cell wall became thick in response to the advancing parasite and eventually stimulated the deposition of a wall apposition or papilla (Fig. 54). The host cell wall was composed of a characteristic thick, double-layered cell wall, even at 20 h after inoculation. At the point of contact, the cell wall of the parasite seemed to merge in with the outer layer of the host cell wall (Figs. 54, 55 and 56). The haustorium was always observed to be surrounded by a thick layer of sheath material (Fig. 60). However the gradual deposition of the sheath can be observed by Figs. 57 and 59 (18 and 20 h) in which the non-interrupted extrahaustorial

Figs. 54-63 Thin section of P. articulatus
challenged by P. virginiana.

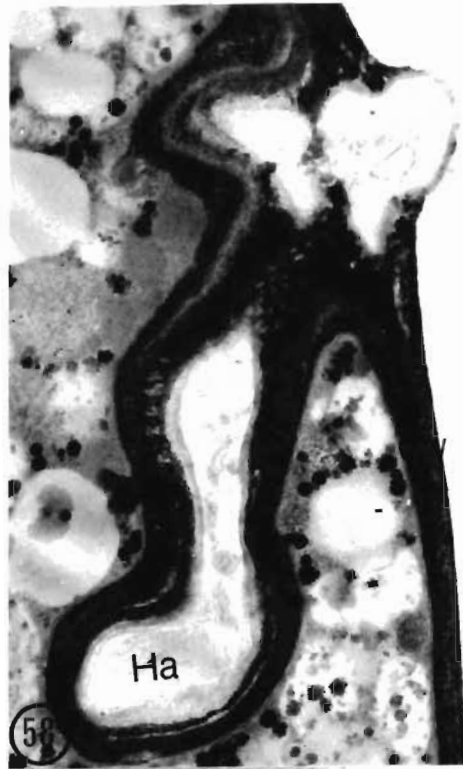
Fig. 54 Early stage of interaction between the
parasite and the resistant host. The
appressorium is attached tightly to
the host cell wall. The outer layer of
the host cell wall is dissolved and
stimulated development of a papilla is
apparent. Note, the thick double layered
host cell wall. X 35000.



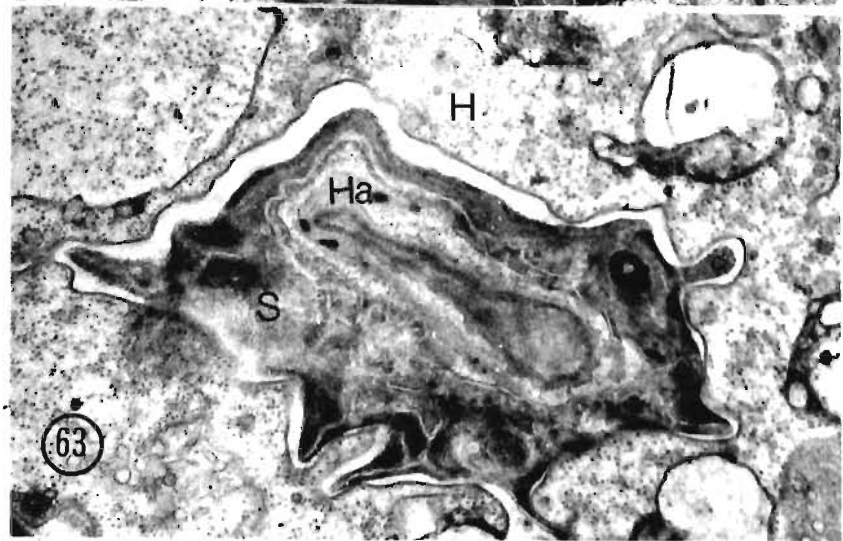
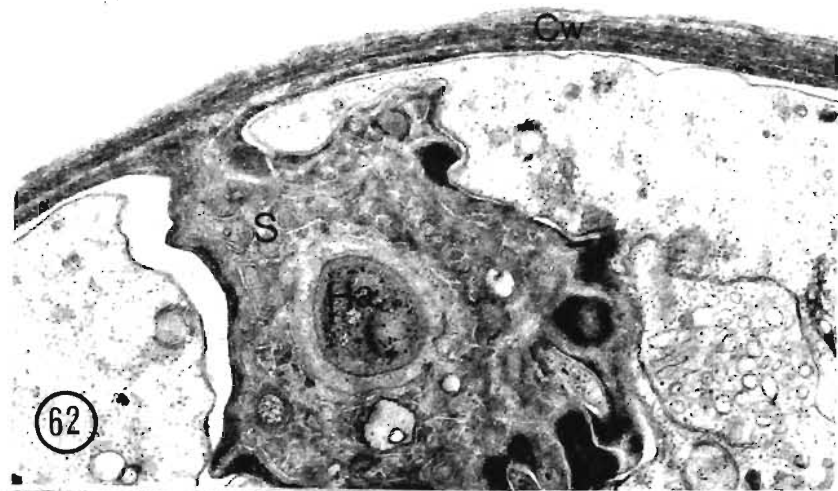
- Fig. 55-56 An initial stage in host parasite interaction. Penetration attempt of the host cell wall by dissolution of the outer layer. The outer layer of the host cell wall appears to partially encase the infection hypha of the parasite. X 20000. In Fig. 56. Note the host cell wall at point of contact is loosely packed as compared to other areas in the cell wall. X 26000.
- Fig. 57 A young haustorium (18 h) within the host cell. Note the presence of a sheath material, surrounded by extra-haustorial membrane. X 28000.



- Fig. 58 An oblique section showing penetration of the host cell and the development of the haustorium. Note an electron-dense sheath around the haustorium. X 15000.
- Fig. 59 A 20 h old haustorium. Note the encasement of the haustorium by extra-haustorial membrane. X 36000.
- Fig. 60 Thin section of a haustorium enclosed in an opaque sheath. X 25000.
- Fig. 61 Electron-opaque blobs (arrow) in the vicinity of the haustorium which in turn is enclosed in a thick encasement. Note the similarity between the sheath material and host cell wall. X 25000.



Figs. 62-63 Young haustoria of P. virginiana,
22 h after inoculation enclosed in
opaque encasement. The haustoria
appear necrotic, without organelles
(compare with Figs. 40-42). X 30000.



membrane is also noticeable. This extrahaustorial sheath appeared to be a continuation of the inner layer of the host cell wall along the protruding haustoria as shown in Figs. 58 and 61. The inner layer of the host cell wall did not appear to rupture, but merely became invaginated by the parasite. Occasionally, large vesicles with the same electron opacity as that of the sheath were observed in the vicinity of the haustorium (Figs. 60 and 61). Numerous haustoria of P. virginiana in the hyphae of P. articulatus were encased completely by enormous aggregates of electron-opaque material (Figs. 62 and 62), which appeared to be a continuation of the inner layer of the host cell wall (Fig. 62). These haustorial lobes were observed to be in stages of apparent degeneration, having lost the integrity of their cytoplasm, becoming dense and absent in organelles hence presumed to be necrotic. This situation was in contrast to the haustoria of the same age produced in C. cucurbitarum (Figs. 40-42, 51-53) and with haustorium produced at 20-22 h in P. articulatus (Figs. 57-59). In no case the haustoria was observed without a thick sheath as was the case in young C. cucurbitarum (Figs. 40-42).

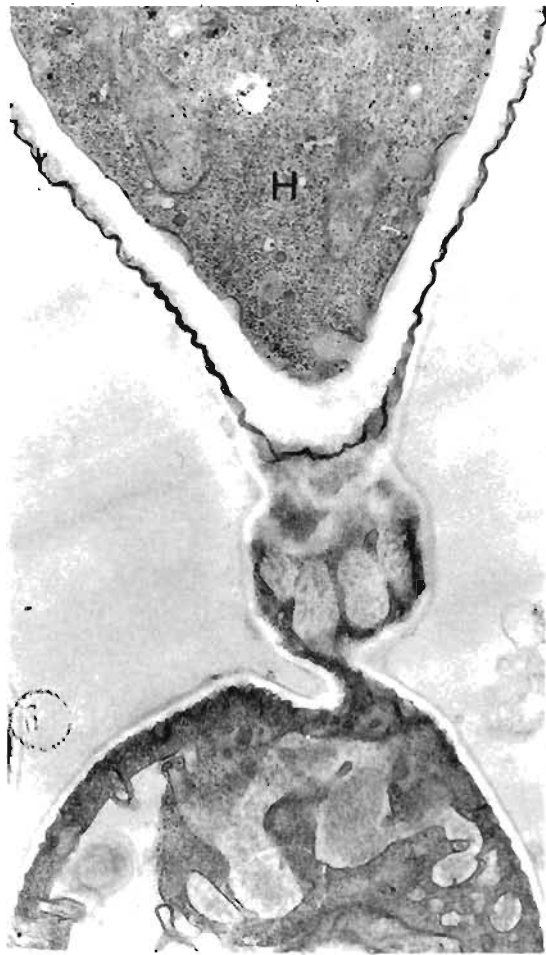
(vii) Interaction Between Piptocephalis virginiana and Non-Host Linderina pennispora

Thorough examination of thin sections cut from different levels of various blocks of P. virginiana when inoculated with L. pennispora, revealed that the parasite did not establish contact with the cell surface of this non-host

species. Fig. 64 shows one of the rare occasions in which the parasite was in close proximity to the host L. pennispora. No change or reaction by the host was noticed on its cell walls in response to the parasite's presence. No indication of attempted penetration by P. virginiana on this non-host species was observed, leading to the assumption that no interaction whatsoever exists between P. virginiana and non-host L. pennispora.

Fig. 64

Thin section of L. pennispora
challenged by P. virginiana. Note
the contact between host and
parasite. The latter made no
attempt to penetrate. X 28000.



CHAPTER 5DISCUSSION

The study of fine structures or electron microscopy has been proven to be a precious tool in resolving the sequence of events in the interaction of host and parasite. However, fundamental to any kind of host-parasite study is an initial assessment of the effects of the parasite on host behaviour, the reactions of the host to the invading parasite and subsequent development of events.

Details of investigations on the parasitism of Piptocephalis virginiana, progressive development of its haustorium, and the host-parasite interface in Choanephora cucurbitarum have been reported previously (Manocha and Lee, 1971; Manocha and Letourneau, 1978). The present study which was intended as a continuation of the investigations of these workers, also included the resistant host species Phascolomyces articulatus and the non-host species Linderina pennispora. No evidence or indication of attempted penetration or contact by P. virginiana of the cell surface of the non-host species, L. pennispora, was observed. This is in accordance with reports by Berry and Barnett (1957), which stated ^{that} the host range of P. virginiana being limited to the members of order Mucorales, and of which L. pennispora is not a member. Furthermore, a different situation was observed when resistant hosts, P. articulatus and old Choanephora cucurbitarum were grown with P. virginiana than that observed in the compatible interaction of P. virginiana and young hyphae of C. cucurbitarum. The

results of compatible interaction in the present study are in full agreement with those of Manocha and Lee (1971) and Manocha and Letourneau (1978), thus indicating that there are dissimilarities in the interaction of these host fungi with their mycoparasite. Piptocephalis virginiana penetrated the young susceptible host by inward push and rupture of the thin single layered host cell wall (Manocha and Lee, 1971; Manocha and Golesorkhi, 1979). Depression of the host cell wall by the infection peg is indicative of mechanical pressure, resembling the infection of Cokeromyces recurvatus by Piptocephalis unispora (Jeffries and Young, 1976) and the infection of higher plants by fungal infection (Politis and Wheeler, 1973). No signs of host cell wall dissolution and digestion at the point of contact and entry of the infection hypha which is generally associated with enzymatic penetration were observed (McKeen et al., 1969; Wheeler, 1975). The ruptured involuted cell wall formed a collar around the haustorial neck. Armentrout and Wilson (1969) did not observe any collar around the haustorial neck of P. virginiana penetrating Mycotypha microspora. The host plasma membrane invaginated along the advancing parasite and did not seem to rupture. A similar situation has been reported for other haustorial parasite-host systems. Continuity of host plasma membrane around the haustoria of Melampsora lini has been reported by Littlefield and Bracker (1970). Abu-Zinada et al. (1975) have also provided information on the invagination of plasma membrane of Vicia faba around the haustorium of Uromyces fabae. The young haustorium was enclosed in its own cell wall and was surrounded

by the host protoplasm. The invaginated host membrane, also called extrahaustorial membrane was adhered closely to the haustorium wall, with a narrow zone of electron opacity, normally accompanying the resistant reactions. It was not until 30-32 h after inoculation that a distinct electron-opaque sheath appeared surrounding the haustorium (Manocha and Lee, 1971; Manocha and Letourneau, 1978). Complete development of the sheath was observed at 36 h after inoculation, which was always coincidental with the development of a secondary cell wall layer. Formation of vesicles in the host cytoplasm in the vicinity of the haustorium, and in the electron-lucent zone were observed, as reported by Shaw and Manocha (1965) which suggested their presence associated with the origin of sheath deposition.

Interaction between the older hyphae of C. cucurbitarum and the parasite P. virginiana was completely different from that of the young hyphae. The cell wall of the old C. cucurbitarum was composed of two distinct layers, differing in electron-density. Appressorial contact of the host cell wall was made through an electron-opaque substance which probably served as a cementing material. Adhesion of the appressorium to the host surface wall is a common phenomenon in Uromyces appendiculatus (Hardwick et al., 1971) and Erysiphe graminis (Edwards and Allen, 1970) where the infection hyphae may disrupt surface particles of wax and attach strongly

(Staub et al., 1974). A similar situation might occur in P. virginiana where apparent unification of the host and the outer layer of the appressorial wall occurs. Most likely this involves structural alterations, indicative of the formation of cementing material which appears to fill the gap between the curvature of the appressorium and the host cell wall. The penetration process of the old hyphae of C. cucurbitarum appeared to be achieved by a combination of both mechanical and enzymatic mechanism (Edwards and Allen, 1970; Ingram et al., 1976; Jeffries and Young, 1978) by the dissolution of the outer layer of the host cell wall at the point of contact and by mechanical push through the inner layer of host cell wall by the parasite. Manocha (1981) through the use of scanning electron microscope was able to show a definite ring zone around the penetration peg of P. virginiana when penetrating the outer layer of the cell wall in a resistant host, P. articulatus. Whereas no change was observed at the point of contact in the cell wall of the susceptible host. Development of a papilla as a response to the parasite was characteristic of the resistant interaction between P. virginiana and old C. cucurbitarum.

In the incompatible interaction, the young haustorium of P. virginiana (18-20 h following inoculation of the 4-5 days old hyphae of C. cucurbitarum) was observed to be enclosed in a distinct electron-opaque sheath. In instances where the parasite managed to penetrate the inner layer of the host cell wall and papilla, it failed to establish direct contact with the host protoplast (contrary to the compatible interaction)

due to the presence of electron-opaque material in the sheath zone. In some cases the electron-opaque sheath around the haustorium appeared to be the continuation of the inner layer of the host cell wall in the older hyphae.

Several investigators have suggested that the age or composition of the cell wall may be a contributing factor to resistance or susceptibility of a species, since there is some evidence that age of mycelium is a factor in the development of the parasite. Berry (1959) recorded that P. virginiana readily attacked young susceptible hyphae of Helicostylum species whereas, the mature hyphae became resistant with age. Karling (1942) reported that Rozella cladochytrii Karling infected young rhizomycelium of its chytrid host more abundantly than the old rhizomycelium. Recently, England (1969) reported that resistance of Phycomyces blakesleeanus to P. virginiana appeared to be principally mechanical and related to age of the hyphae. Young hyphae were heavily parasitized whereas aged hyphae were seldom infected. He further reports that both young and aged mycelium of C. cucurbitarum were heavily parasitized and that age and maturity of the hyphae of C. cucurbitarum had little effect on penetration or subsequent development of P. virginiana. Upon an analysis of young and old hyphal walls of both hosts he reported no significant difference in glucosamine concentration, but that the hyphae of P. blakesleeanus were tougher than those of C. cucurbitarum and resisted breakage when subjected to mechanical pressure. He further concludes that whereas there was little significance

in percentage of wall material in young and old hyphae of C. cucurbitarum, the amount of wall material of P. blakeleenus was many times greater in old hyphae as compared to the young hyphae. He suggests that apparently either the wall composition or thickness is important in mechanical resistance. The results of the present study invalidate his interpretations, since it is obvious now, that there is a difference between the young and old hyphae of C. cucurbitarum in their wall structure (Campbell, 1980) and that age of the mycelium is a factor in the development of the parasite. Furthermore Campbell (1980) has reported a shift in the chitin: chitosan ratio of the wall material in the young and old hyphae of C. cucurbitarum, therefore rendering the interpretations of England (1969) dubious due to lack of insufficient experimentation.

A parallel situation to that of old C. cucurbitarum was observed when a resistant host P. articulatus was infected by P. virginiana. The cell wall of this host was invariably composed of double thick layers, with the young haustorium enclosed in an electron-opaque sheath, which in most cases was continuous with the inner layer of the host cell wall. In some instances the encasing sheath was rather extensively developed with the haustorium devoid of organelles and necrotic. Papilla formation accompanied sites of appressorial contact. Barnett and Binder (1973) reported that haustoria of biotrophic mycoparasites have been observed only in "susceptible" fungus hyphae. This study does not support their view, since haustoria of P. virginiana has been observed in the resistant

host hyphae. Furthermore, Berry and Barnett (1957) reported that P. virginiana was never observed to penetrate the mycelium of a fungus which did not support its growth and suggest that resistance may be due to the failure of the parasite to penetrate the host cell wall. The results of this study are in contradiction to their interpretations. Moreover, the penetration of germinating spores and germ tubes of P. articulatus by P. unispora has been previously described by Jeffries and Young (1978). It is well established from the present study that host resistance in old cultures of C. cucurbitarum and P. articulatus is not due to lack or failure of penetration by the parasite. In this case and in many other cases (Martin, 1964) resistance is expressed only after penetration is accomplished. In general, plant pathogens penetrate moderately resistant plants as readily as they do susceptible ones of the same species. Complete blockage of penetration by highly resistant hosts is probably rare and in many instances penetration is merely retarded rather than blocked. Maize isolates of Colletotrichum graminicola rapidly infects susceptible varieties of maize but produce no visible disease symptoms on oats. On the susceptible host, penetration requires 9 h, whereas on resistant oats, cells are penetrated after 48 h (Wheeler, 1975). This situation is not applicable to the present study since the process of penetration of P. virginiana on both susceptible and resistant hosts require an almost equal time, i.e., 16-20 h. Resistance of old hyphae of C. cucurbitarum and P. articulatus to infection by P. virginiana in the present study is attributed to a lack of direct contact

between the parasite and the host protoplast, which probably prevents the parasite from establishing a nutritional relationship with its host. This contact is prevented mainly by the rapid deposition of electron-opaque material in the space between the haustorium and the host's extrahaustorial membrane, along with the formation of papillae at the sites of penetration. In view of the fine structural studies of these interactions, the results from the light microscopic studies can be interpreted as follows.

The spores of the parasite swell two to three times their original size, upon inoculation with the host fungi. At 15-17 h following inoculation they germinate and produce germ tubes. The germ tubes of the parasite, P. virginiana grow and make contact with the near-by host hyphae. Behaviour of the parasite was similar up to this stage regardless of the nature of the host. However not all host fungi supported equal growth of the parasite under the same conditions. The most rapid and abundant growth of the parasite was observed on C. cucurbitarum. In this compatible host-parasite combination, the parasite penetrates the host and establishes a nutritional relationship with the host and continues to grow to cover the host completely in 3-4 days. In the incompatible host-parasite combination, the parasite penetrates the host hyphae, but its further advance is arrested, as a result of rapid response of host in formation of reaction material. Multiple infections of the resistant host hyphae ^{were} then observed. This phenomenon may have two possible causes. One being the aggressiveness on the part of the parasite. The other more

likely possibility is the failure of the parasite to accomplish a nutritional relationship in the initial attempt, thus forcing it to attempt additional penetrations. In other words, multiple infection of the host hyphae seems to be the result of successive failures on the behalf of the parasite. This situation has been described by Berry and Barnett (1957) in infection by P. virginiana of resistant hosts. At later stages of the infection in the absence of nutrients the parasite weakens and the resistant host outgrows the parasite completely. Furthermore, the result from the germination studies showed that the parasite germinates in the presence of host and non-host species. The comparison of percentage of germinations on the non-host L. pennispora, with those of the host species, suggests that germination inhibitors may not play a significant role in non-host resistance. A similar situation has been described by Heath (1974) in her studies of interactions of host and non-host species with cowpea rust. In another investigation by Mansfield and Hutson (1980) in studying host and non-host responses of broad bean and tulip leaves inoculated with five species of Botrytis they reported little difference between species in their rates of germination under the conditions employed. Investigations on the fine structure of pathogens- plant host interface in compatible and incompatible combinations of wheat stem rust (Manocha, 1975) and flax rust (Coffey, 1976) revealed that the sheath around the haustorium develops earlier in incompatible host-parasite combinations than it does in the compatible combinations. These authors

suggest that incompatibility in these instances should be due to the more rapid deposition of sheath around the advancing haustoria. A similar reaction has been observed more frequently in abortive penetration attempts by rust fungi on resistant host plant (Heath and Heath, 1971). In comparison of the susceptible and immune reactions of cowpea leaves to rust infection; they reported that signs of incompatibility were detected in the immune variety during early stages of haustorial formation when a deposit of callose was formed on the host cell wall at the point of entry of the haustorium, along with the enclosure of haustorium in a sheath. They suggested a slower and more intermittent growth of sheath material in the susceptible reaction. Jeffries and Young (1978) report that papillae formation could be associated with the reactions against parasitic growth of Piptocephalis unispora on the resistant host P. articulatus. They suggest that the papillae prevent the establishment of an active haustoria, or that papillae formation prevents the formation of required numbers of infection needed for active parasitic growth, especially if the continuous proliferation of infection sites is a prerequisite for vigorous parasitic growth. They further support this idea by the fact that penetrations did occur in the thin walled actively expanding regions of the wall of the host and through multiple infections of the resistant hyphae; therefore the parasite, presumably due to absence of nutrients could fail to keep pace with the growth of the host hyphal tips. The results obtained from our germination studies does not support their hypothesis, since no significant difference was observed in

the number of penetrations by the parasite on the susceptible or resistant hosts. Moreover, penetration of the hyphal tip was observed in our system in both the susceptible and resistant hosts. In this study, as well as that of Jeffries and Young (1978) the invading parasite was encountered by the formation of a papillae and extrahaustorial sheath. Papilla are produced by many plants in response to attempted penetration by infectious agents and their deposition as a barrier to the advancing parasite has been reviewed at length by Aist (1976b). Recently interest has developed toward determining their role in plant resistance to attempted fungal penetration (Ride and Pearce, 1979; Sherwood and Vance, 1980). Due to insufficient knowledge regarding their composition, the timing of their production, and their ability to inhibit the growth of an invading parasite, there is little established evidence for their role in plant's resistance to parasites. Employment of autoradiography techniques along with the use of light and electron microscope, which has previously been advantageous in revealing the nature and composition of the sheath zone around the haustorium of P. virginiana (Manocha and Letourneau, 1978) may provide a basis for further critical experimentation required to link such resistance more specifically to papilla formation and provide us with further insight into their induction mechanism, timing of their stimulation and their chemical composition.

In a recent study by Manocha (1981) it was reported that the cell wall composition of host species, C. cucurbitarum

and P. articulatus differed from non-host L. pennispora in the presence of chitosan as the second prominent carbohydrate fraction, next to chitin. The exact role of chitosan in providing attachment sites for the parasite has not yet been established. Furthermore, both host species, C. cucurbitarum and P. articulatus contained γ -linolenic acid, whereas this fatty acid was absent in the non-host species. Manocha and Deven (1975) and (Manocha (1980) have reported a direct correlation between the levels of γ -linolenic acid present in young host and the degree of parasitism by P. virginiana. Therefore, it was concluded by Manocha (1981) that P. virginiana contacts the potential hosts containing chitosan as one of their cell wall components and that its parasitic growth is supported by hosts containing γ -linolenic acid in their cellular lipids. The above mentioned criteria are characteristic of order Mucorales, of which L. pennispora is not a member, which would explain lack of parasitism on this non-host species.

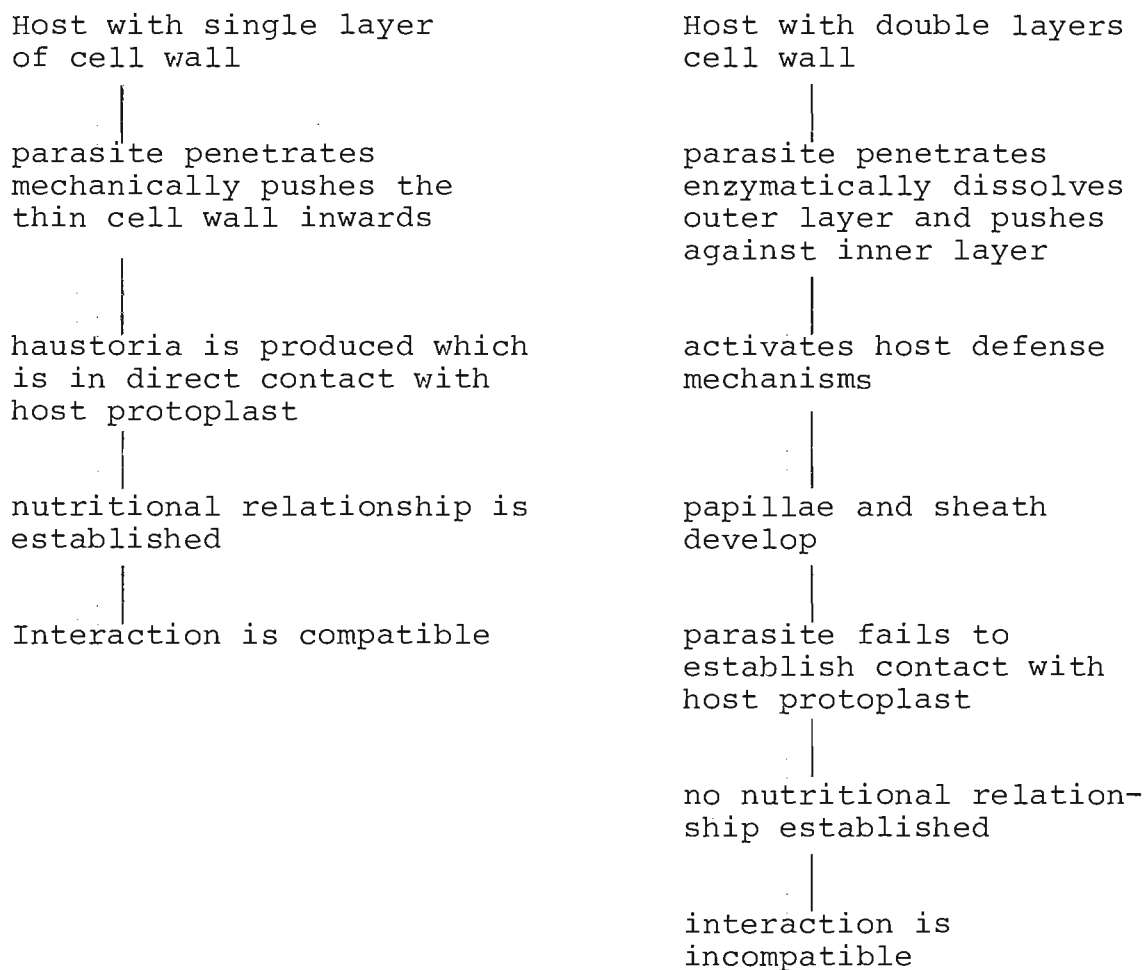
Piptocephalis virginiana is a biotrophic haustorial mycoparasite and does not possess the ability to synthesize some of the nutrients required for its growth and development and is dependent on its potential host which provides those nutrients required by this mycoparasite. However the mere presence of a required nutrient does not necessarily result in susceptibility of the host. In fact most plants are resistant to most infectious agents. Resistance is the rule and susceptibility the rare but economically important except-

ion (Kuč, 1979). Therefore a parasite must be able to absorb the nutrients from the host and not confront resistance by the host (Lewis, 1973). Phascolomyces articulatus is an example of such situation. It is reported to produce γ -linolenic acid but does not support the growth of P. virginiana. The formation of haustorium in this resistant host is opposed by many chemical and physical barriers, some preformed as in the case of double layered cell wall, others like the papilla and sheath are produced in response to infection. A host may invoke several defense mechanisms against a potential pathogen (Heath, 1974). One defense that this resistant host may have at its command is the ability to respond by the process of papilla formation. However the hyphae that do manage to penetrate the papilla are then confronted by other internal mechanisms that restrict the spread of the fungus, such as the sheath encasing of all haustoria in the resistant host. Support of this view can be derived from the occasional necrosis of some haustoria. Question could arise as to which is the initial or most important mechanisms for disease resistance (or susceptibility) in the host. Such arguments may be fruitless in that two or more distinct mechanisms may be operative and the presence of both mechanisms and their coordination may determine the fate of the interaction. Therefore the key to disease resistance in the host may be the functioning of multiple mechanisms for resistance and the main concept is understanding their interaction as one of coordinated defense. Moreover, resistance is often not

dependent on the absolute presence or absence of a resistant mechanism, but rather on the speed and magnitude with which it is expressed. This view can be observed in the case of young hyphae of C. cucurbitarum in which the slow development of reaction material results in its susceptibility to its mycoparasite. Manocha (1981) forwarded a hypothesis on the parasitism of P. virginiana on its susceptible and resistant hosts. He speculates that P. virginiana attempts enzymatic penetration when confronted by a resistant host with two layered cell wall resulting in an incompatible interaction. The enzymatic attempts to penetration by the parasite probably stimulate a defensive reaction in the host by converting the host enzymes from inactive to an active form with the appearance of structural features such as the papillae and the sheath encasement of the haustorium. He further refers to established evidence of conversion of chitin synthetase from an inactive to an active form by proteolytic enzymes, and suggests that since the host cell wall is composed of chitin, it is probable that chitin synthetase activity at the penetration site may be responsible for the formation of papilla and rapid development of sheath material therefore preventing the parasite from establishing a direct contact with the host protoplast. On the same token, the initial contact with the young susceptible host might have suppressed the enzyme production of the parasite, forcing it to use mechanical means for penetration. Manocha's hypothesis (1981) on parasitic behavior of P. virginiana on susceptible and

and resistant hosts is summarized in the following figure:
(Fig. 65).

Hosts with γ -linolenic acid and chitosan



Those interactions reflecting the inability of host and parasite to co-exist are termed incompatible, in which the host's resistance depends on its ability to prevent or restrict the establishment and subsequent activities of the potential

parasite. Whereas, interactions in which the parasite ramifies within the host and in the absence of those reactions observed in the incompatible association are referred to as compatible. The latter of course usually results in hosts susceptibility (Daley, 1976). What transpires at the very initial interaction of host and parasite determines in most cases the fate of the association between the two members. If all systems are functional a susceptible interaction occurs, if all systems are negative resistance is established, therefore the details of the chemical structures that interact at the surface of both host and parasite should be uncovered (Sequeira, 1979). In the mycoparasitic system reported by Manocha (1981) the precise role of chitosan and/or any other sugars in providing attachment sites for the parasite has not been elucidated. However, his study on the structural aspects of the cell walls did show some promise. The cell wall of the host is a barrier to penetration which the parasite must first overcome in order to establish a nutritional relationship with the host, therefore its structure and/or composition will determine the outcome of the relationship, i.e., success or failure of parasitism (Brian, 1976). The results of an earlier study by Manocha and Letourneau (1978) providing unequivocal evidence that the haustorial sheath is composed of host cell wall material, and of the present study showing continuation of the inner layer of the host cell wall into the sheath, along with a double layered host cell wall in the resistant interactions, clearly suggests that the host cell wall plays a significant

role in resistance to the haustorial mycoparasites. Furthermore, the different mechanism of penetration by P. virginiana in the young and the old C. cucurbitarum probably depends on the chemical composition of the host-surface constituents. The cell wall is a dynamic component of the host cell in C. cucurbitarum and it seems to alter with age. Further investigations on the details of molecular architecture of the surface of walls of the young and old hyphae of C. cucurbitarum may prove useful in further elucidating the mechanisms of resistance and susceptibility of this host to its mycoparasite P. virginiana.

CONCLUSIONS

The results of the present study show that there are three patterns of behaviour of Piptocephalis virginiana towards its potential hosts and non-host fungi which are as follows:

First, the germ tubes of the parasite contact the host hyphae, appressoria and haustoria are produced and parasitic growth develops (as the case with young Choanephora cucurbitarum). Secondly, although the infection apparatus develops further growth of the parasite is curtailed and the resistant hosts, Phascolomyces articulatus and old Choanephora cucurbitarum outgrow the parasite.

Thirdly, when grown with a non-host species, Linderina pennispora, no indication of penetration by the parasite was observed and the parasite continued its growth across this non-host.

However, the parasite germinates equally well in the presence of host and non-host species suggesting that probably no inhibitory factor(s) is involved. This implies that fundamental differences in the nature of these parasitic associations with susceptible, resistant, and non-host species do exist. Morphological events occurring during and after haustorium formation may thus be involved in the resistance of P. articulatus and old C. cucurbitarum to parasitism by P. virginiana.

The host cell wall plays a definitive role in resisting

the establishment of the parasite. The cell wall of the young susceptible host, C. cucurbitarum is composed of a single layer, and mechanical penetration of this host results in a compatible interaction. Resistant hosts, P. articulatus and aged C. cucurbitarum possess a double layered cell wall. The penetration of the resistant host appears to be by enzymatic dissolution of the outer wall layer. The failure of the parasite to establish a nutritional association with the resistant host may either be due to the papilla formation by the hosts in response to invasion by the parasite or to the rapid development of an extensive sheath around the invading parasite, thereby preventing the direct contact with host protoplast. The results of a previous study by Manocha and Letourneau (1978), providing unequivocal evidence that the haustorial sheath is composed of host-cell wall material and the present study showing continuation of the inner layer of the host cell wall into the sheath, clearly suggest that the host cell wall is responsible for its resistance to this haustorial mycoparasite. Furthermore, the mechanism of penetration by P. virginiana is different in the young and the old C. cucurbitarum, probably depending upon the chemical composition of the host-surface constituents. Further investigations on the molecular architecture of the walls of the resistant hosts may prove useful, in precisely elucidating the mechanisms of resistance and susceptibility of these host to their mycoparasite, P. virginiana.

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APPENDIX I

Percentage germination and penetration of spores of *P. virginiana*
inoculated with spores of *C. cucurbitarum* (MEXE. 18 h).

Total number of spores	Spores germinated	Spores penetrated	Spores swollen	Spores not germinated	% spore germination	% spores penetrated
189	151	69	20	18	79.9	36.5
191	146	68	22	23	76.4	35.6
187	149	67	22	16	79.7	35.8
168	125	59	31	12	74.4	35.1
176	134	67	28	14	76.1	38.0
163	116	62	26	21	71.2	38.0
146	103	55	29	14	70.5	37.6
128	96	46	16	16	75.0	35.9
145	104	51	26	15	71.7	35.1
187	142	60	26	19	75.9	32.0
158	127	59	17	14	80.4	37.3
151	126	63	13	12	83.4	41.7
94	87	63	--	7	92.6	67.0
81	75	53	--	6	92.6	65.4
73	68	47	--	5	93.2	64.3

APPENDIX II Percentage germination and penetration of spores of P. virginiana inoculated with spores of P. articulatus (MEXE, 18 h)

Total number of spores	Spores germinated	Spores penetrated	Spores swollen	Spores not germinated	% Spores germinated	% Spores penetrated
78	60	27	10	8	76.9	34.6
65	56	21	7	2	86.1	32.3
82	79	41	3	-	96.3	50.0
141	103	44	13	25	73.0	31.2
138	114	39	14	10	82.6	28.2
127	100	40	15	12	78.7	31.4
144	100	56	26	18	69.4	38.8
137	98	52	25	14	71.5	37.9
142	108	52	22	12	76.0	36.6
134	101	42	20	13	75.4	31.3
119	92	47	18	9	77.3	39.4
100	82	22	10	8	82.0	22.0
68	51	19	--	17	75.0	27.9
71	67	36	--	4	94.4	50.7
53	50	29	--	3	94.3	54.7

APPENDIX III Percentage germination of spores of P. virginiana inoculated with spores of non-host species L. pennispora (MEXE, 18 h)

Total number of spores	Spores germinated	Spores swollen	Spores not germinated	% spores germinated
119	55	21	43	46.2
128	68	28	32	53.1
138	71	22	45	51.4
123	67	25	31	54.4
108	55	16	37	50.9
121	49	29	43	40.4
140	53	31	56	37.8
176	86	24	66	48.8
142	59	32	51	41.5
108	24	32	52	22.2
136	65	35	36	47.7
107	52	29	26	48.5
111	58	11	42	52.2
117	66	19	32	56.4
151	102	18	31	67.5